Characterisation of proteins in camel milk, the effect of heat treatment on physicochemical and functional properties related to yogurt

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ABSTRACT

Camel milk plays a central role in the food supply of Eastern African and Middle Eastern countries (e.g. Kenya, Somalia, Saudi, and Ethiopia), home to the majority of the world's camel population. Raw, and traditionally fermented camel milk has become increasingly commercialised and consumed in urban areas. This has led to an increased interest in the processing of camel milk for the urban market, with skimmed camel milk casein and whey powders soon to be commercialised, in a manner similar to the already widely available bovine dairy powders. However, little information is currently available concerning the effects of different processing methods (e.g. thermal treatment) on camel milk fractions. Currently there are no camel milk derived products, such as yoghurt or cheese, available in local Saudi supermarkets. Furthermore, the abundance of bioactive substances in camel milk have been reported to have useful effects; one of the most prominent is the anti-diabetic benefits revealed by *in vivo* studies. However, the presence of insulin in camel milk still remains to be proven.

The aims of this thesis are twofold. The first area (Chapters 2, 3 and 4) describe the effect of heat treatment on camel milk components and their functional properties in an oil and water emulsion, and in yoghurt. In order to test these functional properties, camel skimmed milk, whey and casein were prepared and freeze dried. The key novel findings include: skimmed milk that had been heat treated and freeze dried showed significantly improved functionality for use in emulsions and yoghurts, whereas heat treated whey did showed no significantly enhanced functional properties. Furthermore, non-heated freeze dried casein significantly enhanced curd formation in yoghurt, and resulted in a smooth texture. Two fermented yoghurts were developed containing heat treated skimmed milk powder or casein with similar textural properties to bovine milk commercial yoghurt, that were acceptable in taste and texture, as determined by an independent study for sensory evaluation. The second area of this study (Chapter 5) concerned the characterisation of insulin in camel milk. As it was confirmed by previous studies that the presence of high concentration of insulin in camel milk comparing to bovine milk. Key findings were that no protein with a characteristic molecular weight similar to bovine or human insulin (5.8 KDa) could be detected using Western Blotting; however, a 62 KDa protein showed consistent immune reactivity. ELISA results showed high immune reactivity in camel whey. An *in vivo* assay showed

biological insulin like activity in camel milk, but the validity of the assay still needs to be confirmed.

DECLARATION

I, Alyaa munaji Homoud, hereby declare that I am the author of this thesis. All the work described in this thesis is my own except where stated in the text. Results presented in this work have not been used in any previous application for a higher degree. All sources of information have been consulted by myself and are acknowledged by means of references.

Alyaa munaji Homoud

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CAMEL MILK: GENERAL INTRODUCTION

1.1 Camel Classification and Evolution

A camel is an even-toed ungulate within the genus *Camelus* bearing unique fatty stores known as "mounds" on its back. The two surviving types of camel are the dromedary, or one-humped camel (*C. dromedarius*), which is found in the Middle East and the Horn of Africa, and the Bactrian, or two-humped camel (*C. bactrianus*), which is found in Central Asia (Mburu et al., 2003). The two-humped Bactrian camel (Figure 1, Pic 3) lives in cold climates, whilst the one-humped dromedary (Figure 1, Pic 6) lives in hot climates and is adapted to desert conditions. Both species have been domesticated; they provide milk, meat and hair for materials or merchandise, such as felted pockets, and are working animals whose uses range from human transport to burden-bearing.

The term "camel" is used extensively to refer to any of the six camel-like species belonging to the family Camelidae. This comprises the two genuine camels, the dromedary and the Bactrian, the four South American camelids, the llama and alpaca, known as the "New World camels" and the guanaco and vicuña, known as the "South American camels" (Figure 1). The most remarkable feature of camels is their ability to continue to lactate milk with a high water content (up to 90%) even when dehydrated (Wernery, 2006).

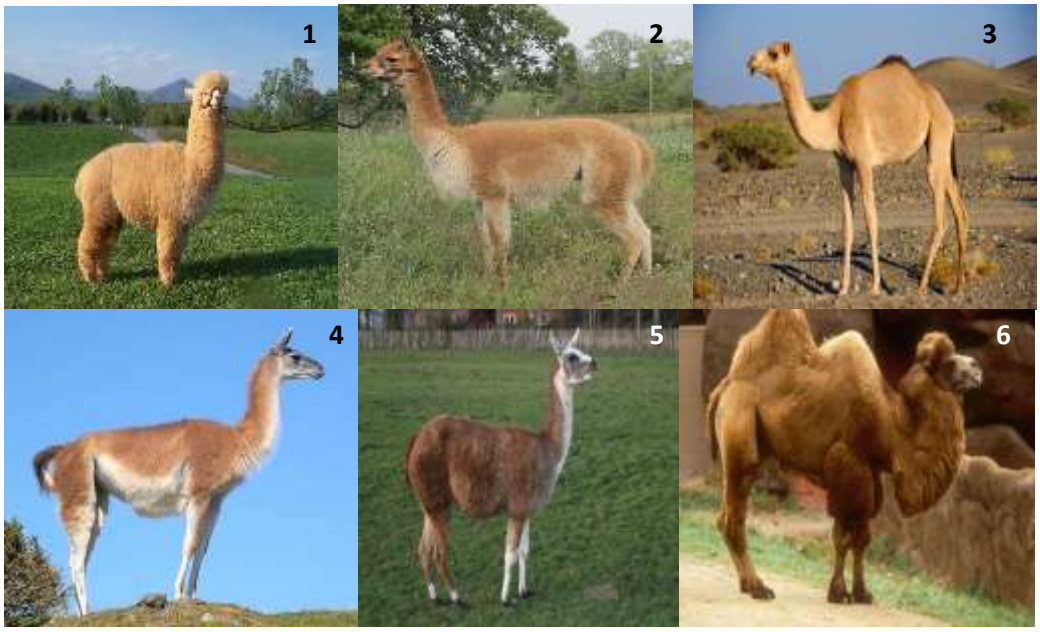

Figure 1 Six camel-like highly-evolved species in the family Camelidae

1. Alpaca 2 Vicuña 3 Dromedary 4.Guanaco 5.Llama 6. Bactrian

The dromedary camel is familiarly known as “the ship of the desert” (Dikecc, 2002). The lack of water and food (Traore et al., 2014) and other desert conditions comprise a harsh environment, but camels are naturally adapted to these conditions and are able to live quite easily in the desert. Moreover, they are able to provide plenty of milk, more than any other species, and for longer periods of time (Zeleke, 2007; Mace, 1998; Sadler et al., 2010). Lactating camels each produce between 1,000 and 12,000 litres of milk for anywhere between 8 and 18 months. The world’s biggest camel milk producer is Somalia, with 850,000 tonnes a year, followed by Saudi Arabia with 89,000 tonnes (FAO. 2012). Many camels belong to desert families who distribute the milk locally to family members rather than send it to cities, as they usually live a long way from commercial markets. The taste of camel milk is quite acceptable; it is usually sweet and sharp, but may be salty as a consequence of the type of plants the camel has been exposed to and the lack of water (Yagil and Etzion, 1980).

1.2 Camel population in African countries and the rest of the world

According to Ramet (2001), the total world population of camels is estimated to be about 20 million. African countries have the highest proportion of the worldwide camel population. Somalia has the largest herd (Shabo et al., 2005; Farah et al., 2007), which is recorded as producing an average monthly milk yield of approximately 150 litres (Angoh, 1997).

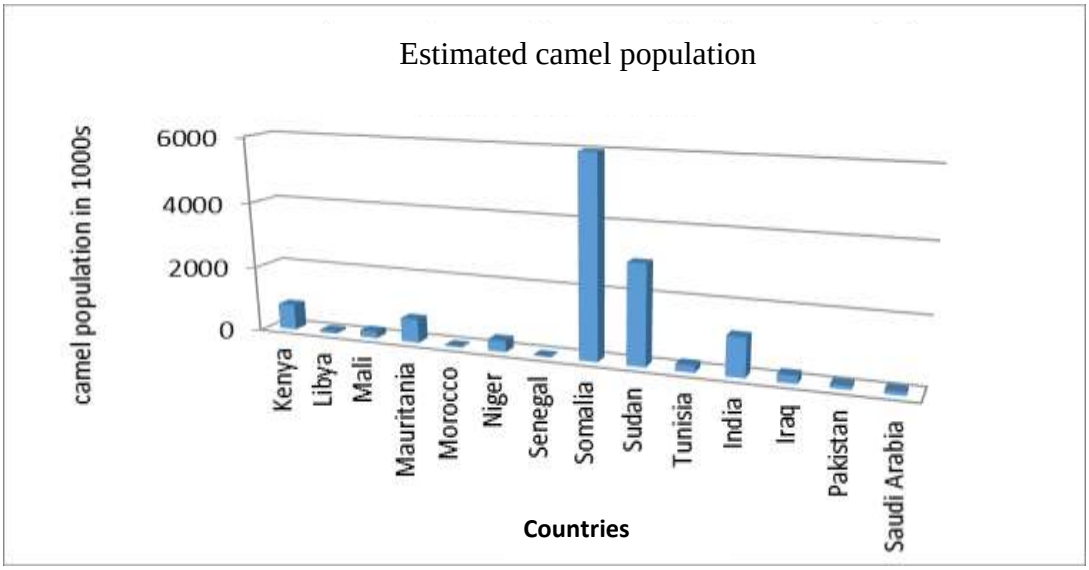

Figure 2 Approximate camel population in African countries and the rest of the world. (Ramet,2001).

1.3 Applications of camel products

1.3.1 Camel as a food source

Many pastoral groups and communities in a diverse range of ecosystems around the world depend on camel meat and milk for their livelihood (Sweet, 1965; Agab and Darosa, 2007). Camel milk is widely used in different countries and regions, including India, Africa and the Middle East. Camel milk is not available to the population in local supermarkets as the majority of camels are owned by tribal groups in desert zones (Mal and Pathak, 2010). In Dubai and some areas of Saudi Arabia, some dairy products made from camel milk, such as ice cream, cheese and chocolate, have become available in supermarkets (Wernery 2006).

Camel milk is a staple of desert migrant tribes; a traveller can live on camel milk alone for almost a month. Bedouins (Arabic tribes) know that the beneficial effects of camel milk are improved if the camels' diet includes certain desert plants. Camel milk can be made into drinkable yoghurt (cultured milk), (Bekele et al., 2011).

One study carried out in the Emirates investigated the desirability of camel milk to children in elementary school. A panel of 173 students was selected to evaluate several milk samples (fresh cow's milk, dried cow's milk, fresh camel milk and chocolate-flavoured camel milk). A seven point hedonic scale (using smiling faces) was used to rate the colour, aroma, taste, texture and overall acceptability of the milk. Camel milk scored the lowest ratings for taste (Hashim, 2002).

Table 1 Sensory evaluation for camel and bovine milk (results out of 10).

Sample	Taste	Aroma	General
Camel milk	3.2	4.1	3.8
Bovine milk	5.8	5.9	5.8
Dried bovine milk	5.3	5.7	5.5
Chocolate camel milk	6.4	6.3	6.2

Camel milk cheese

Camel milk cheese has not yet been commercially produced due to poor coagulation properties. Some additives have been reported to improve the coagulation stage, such as Camifloc, a special enzyme for coagulation (Clemmesencl, 2008). Clemmesencl was

the first to use Camifloc for making camel milk cheese in Africa, specifically Mauritania, as reported by Willensky (2011). Furthermore, investigation of chymosin and yoghurt starter culture (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) has found that 1.7 ml of chymosin/L of milk is the optimal quantity required to make camel cheese (Benkerroum et al., 2011).

Many types of cheeses could be made from camel milk. Mehaia (1993) experimented with various manufacturing procedures and compositions of fresh soft white cheese made from camel milk. Different percentages of fat, salt, and two lactic starter cultures were used (Mehaia, 1993). Sensory evaluation by a taste panel was conducted to determine the acceptability of the cheeses. The cheeses made from milk (1.5% fat and 3% salt) with lactic starter cultures were the most acceptable, whereas the least acceptable were those made from whole milk (3.9% fat) and 0 or 1% salt (Mehaia, 1993). However previous studies of camel milk have not yielded sufficient information about the functional properties of camel milk. Further research into camel milk is required to improve the quality, test, curd and the yield of this type of cheese, as recommended by Mehaia (1993) and Ramet (2001).

Meat

A camel cadaver can provide a significant amount of meat. The male dromedary body can weigh 300 to 400kg, while the cadaver of a male Bactrian can weigh up to 650kg. The brisket, ribs and loin are among the most favoured parts, and the protuberance is viewed as a delicacy. The mound contains “white and debilitated fat”. Camel meat is coarse and the meat of older camels can be exceptionally tough, although camel meat becomes more tender the longer it is cooked (Kadim, 2013).

Camel meat has been eaten for quite a long time and has a number of benefits. It is low in fat, highly nutritious and has the potential to be used to combat hyperacidity, hypertension, pneumonia and respiratory disease (Kadim, 2013). Camel meat is still consumed in some countries, including Eritrea, Somalia, Djibouti, Saudi Arabia, Egypt, Syria, Libya, Sudan, Ethiopia, Kazakhstan and other dry areas where sources of protein may be restricted or where the consumption of camel meat has a long social history (Kadim, 2013).

Wool and hides

Wool is an important camel by-product in many camel-producing countries. The average wool clip is 3.28kg for males and 2.10 kg for females. Fibre diameter is 12 to 27 microns and the length ranges from 4 to 12 cm. Dromedary wool has a number of valuable technological properties, such as low heat conductivity, softness and strength. A wide range of warm fabrics are manufactured from camel wool.

In the tanning process, the hide needs to be split in half so that the hump section can be flattened to enable tanning through commercially available machines. Hides have been tanned both as "Fur On" to form skins and "Fur Off" as leather (O'Hora, 2014).

1.4 Medicinal properties of camel milk

Many health problems have been reportedly treated by camel milk. There is a great deal of truth in the quotation from the Qur'an, the Muslim holy book, which states that "the camel was given to man as a gift from God". Camel milk is low in cholesterol (Gorban and Izzeldin, 2001; Al-Numair et al., 2010), rich in Na, K, Zn, Fe, Cu, Mn, niacin and vitamin C content and relatively low in thiamine, riboflavin, folacin, vitamin B12, pantothenic acid, vitamin A, lysine and tryptophan compared to bovine milk (Sawaya et al., 1984).

Recent reports indicate that camel milk may have potential health benefits, including hypocholesterolaemic, hypoglycaemic, antimicrobial and hypoallergenicity effects (Al Haj and Al Kanhal, 2010) and may have anti-carcinogenic, anti-diabetic, and anti-hypertensive properties (Shabo et al., 2005). Camel milk is reported to have medicinal properties that could help to treat several illnesses, including tuberculosis, ulcers, respiratory ailments and hepatitis (Meiloud et al, 2011; Al-Ayadhi et al., 2013).

Camel milk is stable at room temperature (up to 30^{o}C) for more than eight hours before turning sour (pH 5.8), whereas bovine milk turns sour after just three hours at the same temperature (pH 5.7). This might be the result of the high content of antimicrobials present in camel milk, such as lactoferrin and immunoglobulin (Haj and Kanhal, 2010; El-Hatmi et al, 2007).

Camel milk can be used as a substitute for bovine milk for children who are allergic to the latter. One study investigated the effect of consuming camel milk on eight children with severe food allergies who had not benefitted from conventional treatments for their allergies. It was found that none of the eight children reacted negatively to the camel

milk and their allergy to the proteins was reported to be very low when compared to their allergic reactions to protein in bovine milk (Agrawal et al. 2004; Wernery 2006).

The whey milk fraction has been used as a traditional treatment for many common health problems caused by microorganisms as it contains high concentrations of natural antimicrobial and antiviral compounds such as lactoferrin and lysozyme (Marshall, 2004). They are highly temperature resistant (Elagamy, 2000).

Another study used β-casein to investigate the anti-oxidant activity after hydrolysis by digestive system enzymes. After enzymatic hydrolysis, both antioxidant and ACE-inhibitory activities (Angiotensin-Converting-Enzyme inhibitor) of camel whole casein and camel β-casein were enhanced (Salami et al., 2011).

1.4.1 Treatment of Lactose Intolerance

In normal circumstances, the human body digests and absorbs lactose in the intestine using the enzyme lactase, which is located on the surface of the cells that line the small intestine. The enzyme splits the lactose into glucose and galactose, which are then absorbed into the bloodstream through the intestinal wall (Carrigan, et al., 2010).
Lactose intolerance is a common digestive problem in which the body is unable to carry out its digestive functions because of insufficient lactase. Lactose-intolerant people can therefore suffer from a lack of calcium, particularly children who need it most. Camel milk has become an attractive alternative for such people. Lactose intolerance against camel milk, for unknown reasons, does not exist (Cardoso et al., 2010 and Shabo et al., 2005)

1.5 Novel application for camel casein

Research has been carried out into camel casein, particularly with β-casein, to investigate the possibility of using it in a capsulized curcumin substrate, a potent anticancer and antioxidant natural polyphenol that is poorly soluble in aqueous solutions (Esmaili et al., 2011). Esmaili et al. (2011) used camel β-casein for curcumin encapsulation the presence of camel β-casein increased the solubility of curcumin at least 2,500-fold.

1.6 Camel milk composition and nutritional value

1.6.1 Chemical properties of the milk

Farah (1993) examined the different sources of freeze-dried camel milk compositions

The largest differences were found in dry matter. This was caused by the difference in the concentration of water before the freeze-drying stage. The differences between the various datasets reflect differences in the breed and lactation state of the animals and the sampling procedures and analytical procedures used. Water concentration is also an important factor that affects the total solid composition of camel milk (Yagil and Etzion, 1980).

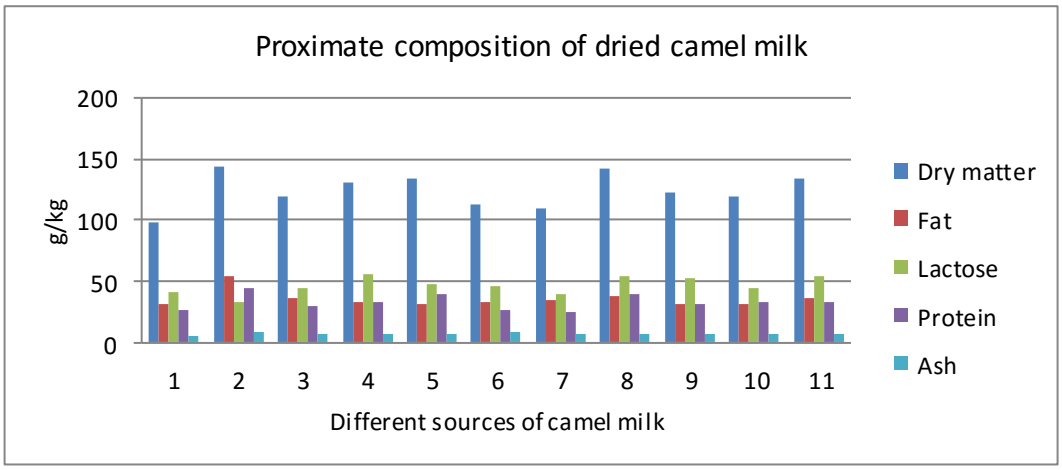

Figure 3 Composition of dried camel milk obtained from different sources (g/kg) (Farah, 1993).

The most important factor affecting the overall composition of camel milk in its liquid form is water content. It has been clearly demonstrated that experiments that restricted drinking water caused an decrease in water content and a subsequent increase in total solids (Ramet, 2001).

Table 2 Chemical composition of bovine and camel milk in its liquid form (Khan and Iqbal, 2001; O'Connor, 1995)

%	Bovine milk %	Camel milk %
Protein	2.9 to 5	2.7 to 4.5
Fat	2.5 to 6	2.9 to 5.2
Lactose	3.6 to 5.5	3 to 5.5

The composition of camel milk whey is characterised by higher total solids than bovine milk (7.0 and 6.5 percent respectively),

The small size of fat globules and the fragility of the casein micelle network are the cause of these losses (Ramet, 2001).

1.6.1.1 Camel milk nutritional value

1.6.1.1.1 Camel milk minerals and vitamins

Konuspayeva et al. (2011) found that camel milk contains considerably less vitamin A and B2 than bovine milk, while the level of vitamin E is about the same. The level of vitamin C in camel milk is on average three times higher than that in bovine milk **(K**onuspayeva et al., 2011).

Table 3 The most important vitamins in camel milk compared to bovine milk. (Park and Haenlein, 2006; Bonnet, 1998; Fox, Fuquay and McSweeney, 2011; Yasmin et al., 2012; Ramet, 2001)

Vitamins mg/kg	Camel milk	Bovine milk
C	24 - 52	3.23
B1	0.33-0.60	0.28 - 0.90
B2	0.42-0.80	1.2 - 2
Niacin	4-6	0.5 - 0.8
B12	0.002	0.002 - 0.007
B6	0.52	0.40 - 0.63
Folacin	0.004	0.01 - 0.10
A	0.050 I.U	0.150 IU

Camel milk has relatively high niacin and vitamin C, and relatively low thiamine, riboflavin, folate, vitamin B12, pantothenic acid and vitamin A compared to bovine milk (Nikkhah, 2011). Camel milk has a very high percentage of ascorbic acid (vitamin C) when compared to other types of milk sources (Mohamed et al., 2005); the concentration of vitamin C is between three and five times greater than that in bovine milk (Agrawal et al. 2004; Wernery., 2006). However, the vitamin C in camel milk is significantly more sensitive to heat than that in bovine milk (Mehaia et al 1994)

Table 4 Vitamin C concentration in milk types.(Farah, Rettenmaier and Atkins, 1992; McSweeney and Fox, 2009).

Milk type	Mg/kg
Bovine	2.11
Goat	5.48
Human	3.02
Camel	6.2

Table 5 The mineral composition in camel and bovine milk. (Haddadin et al., 2008; Al-Awadi and Srikumar.,2001; Cashman, 2006; Dell'Orto et al. 2000; Gorban and Izzeldin, 1997).

Minerals	Camel milk	Bovine milk
Sodium	690 mg/l	520mg/l
Zinc	5.8mg/l	3.9mg/l
Iron	4.4mg/l	0.5 mg/l
Manganese	0.05mg/l	0.03mg/l
Phosphorus	715 mg/l	890mg/l
Calcium	1.9 g/l	1.120 g/l
Potassium	1.703 g/l	1.36 g/l

Camel milk has a greater amount of Na, K, Zn, Fe, Cu and Mn than bovine milk.. Camel milk is a good source of iron for people with an iron deficiency disorder.

1.6.1.1.3 Fatty acids and camel milk lipids

Milk fat contains approximately 400 different fatty acids, making it the most complex of all natural fats (Maansson, 2008).

The total fat content is 32.8 g/L ± 14 in camel milk. Triacylglycerol accounted for 96% of the total lipids in milk. Triacylglycerol of camel milk contained saturated and unsaturated fatty acids at a concentration of 66.1% and 30.5% respectively. There are 16 predominant saturated fatty acids: (34.9%) 14: 0 (14.5%) and 18: 0 (9.7%). The ratio of unsaturated/saturated acid is more favourable in camel milk compared with that of

bovines or other mammals. It is more similar to goat's milk and contains fewer short-chain fatty acids than bovine, sheep or buffalo milk (Nikkhah, 2011).

Table 6 Fatty acid composition (w/w%) of camel, human and bovine milk (De Marchi et al., 2011; Collomb et al., 2001; Shamsia, 2007; Yassir et al., 2010).

Fatty acid common name	Fatty acids	Camel milk	Bovine milk
Butyric	C4:0	0.76	1.6
Caproic	C6:0	0.32	2.46
Caprylic	C8:0	0.19	0.41
Capric	C10:0	0.26	1.09
Lauric	C12:0	0.45	1.36
Myristic	C14:0	9.9	4.27
Palmitic	C16:0	27.2	10.52
Stearic	C18:0	13.6	3.28
Arachidic	C20:0	0.9	0.03
Unsaturated fatty acids			
Myristoleic	14:01	1.6	1.26
Palmitoleic	16:01	12.3	1.47
Oleic	18:01	26.2	23.91
Linolelaidic	18:02	4.8	2.53
α linolenic	18:03	1.5	0.38

1.6.1.1.4 Lactose in camel milk

Lactose is a reducing sugar; it is a disaccharide sugar present in milk and other dairy products. It comprises two smaller simpler sugars, glucose and galactose.

Table 7 The quantity of lactose in different types of milk (Carrigan, et al., 2010).

Milk	g/100g
Bovine	4.6
Human	7.2
Camel	4
Sheep	5.1

1.6.1.1.5 Amino acid composition

Table 8 The amino acid composition of camel, bovine and human milk (g/100g of protein) (Shamsia, 2009; Sabahelkheir et al., 2012; Park and Haenlein, 2006).

Amino acids	Camel milk	Bovine milk
Alanine	3.3	5.1
Arginine	5.1	3.9
Aspartic	7.2	10.7
Cysteine	1.5	0.9
Glutamic	21.1	18.6
Glycine	1.2	2.3
Proline	13	14
Serine	3.0	5.9
Tryptophan	1.8	1.4
Tyrosine	3	0.3
Essential amino acids		
Histidine	2.9	1.4
Isoleucine	4.9	7.3
Leucine	9	19
Lysine	6.6	8.1
Phenylalanine	2.6	1.1
Methionine	3.7	7.9
Threonine	5.3	2.7
Valine	4.8	8.1

Amino acids are organic compounds that contain both an amino group and a carboxyl group. The human body can synthesise all of the amino acids necessary to build proteins, except for the “essential amino acids”. An adequate diet must contain these essential amino acids. Typically, they are supplied by meat and dairy products (Smolin and Grosvenor, 2008).

Camel milk has lower concentrations of essential amino acids leucine, isoleucine, leucine, lysine, methionine and valine than bovine milk. (Salmen et al., 2012).

1.7 General Objectives

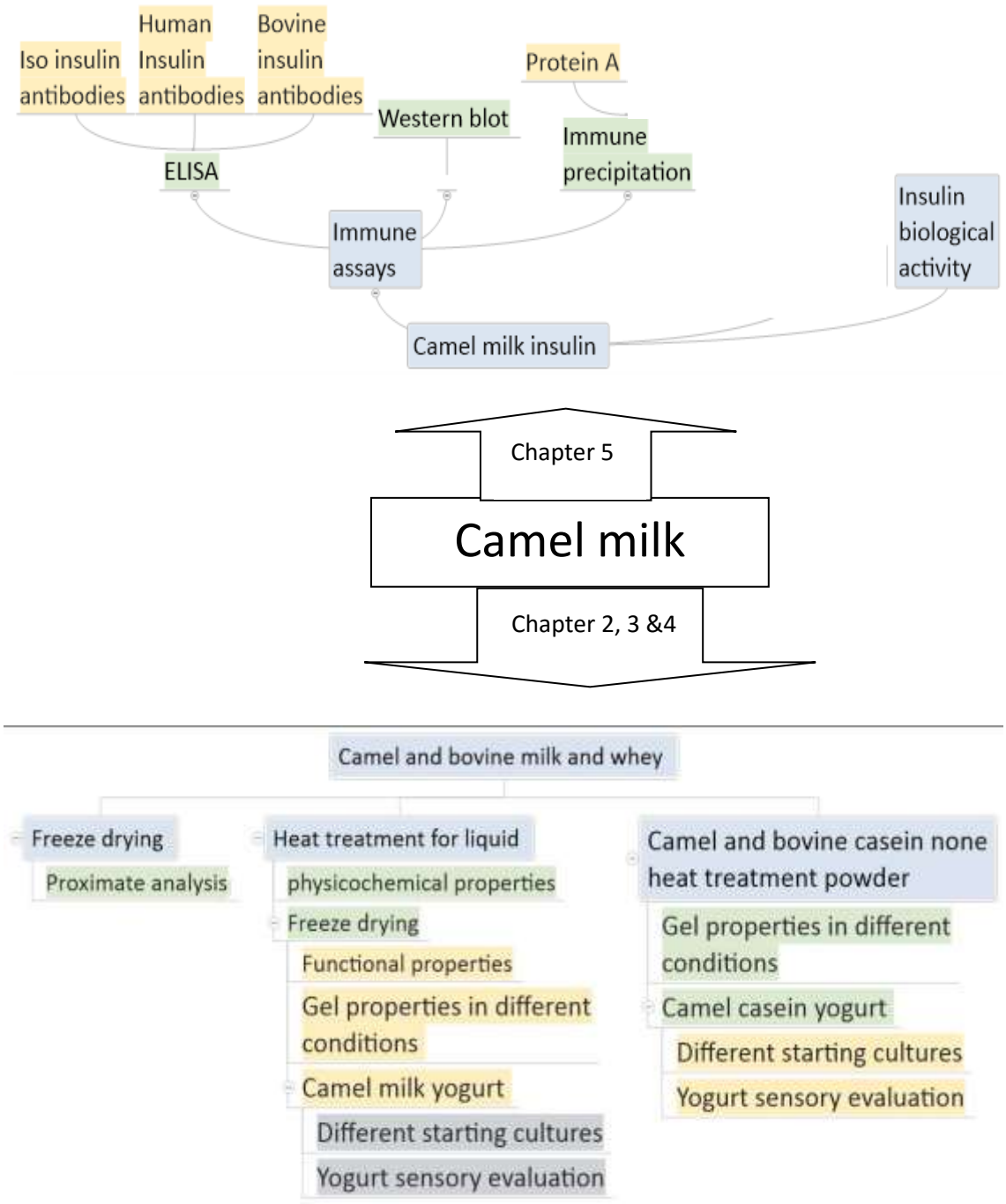

Figure 4 General project aims

Objectives

The main objective of studying camel milk properties was to provide the saudi dairy industries with the fundamental information for processing camel milk into products (chees or yogurt) . Moreover, investigate the potential of camel milk protein that provide the similar affect of insulin to the blood glucose.

1.7.1 Effect of heat treatment on physicochemical and functional properties

1.7.1.1 Aims

- To measure the effect of heat treatment on liquid skimmed camel milk and whey, in comparison to bovine milk and whey at neutral pH.
- To prepare skimmed milk powder, whey powder and casein powder from camel milk and bovine milk.
- To determine the proximate composition of camel and bovine skimmed milk and whey powder.
- To determine the functional properties of proteins in an emulsion prepared with preheated camel and bovine milk powder fractions.

1.7.1.2 Novelty of the study

- The effect of heat treatment on turbidity, total sulfhydryl groups and glycation of skimmed camel milk has not previously been reported.
- The effect of heat treatment on the solubility and emulsifying properties of skimmed camel milk at neutral pH has not previously been reported.
- The effect of heat treatment on turbidity, free and total sulfhydryl groups and glycation of camel whey protein has not previously been reported.
- The effect of heat treatment of camel whey on solubility and emulsifying properties at neutral pH has not previously been reported.

1.7.3 GDL Gels

1.7.3.1 Aims

To develop an acid gel using GDL to serve as basis for the development of a fermented yoghurt

1.7.3.2 Novelty

The effect of adjusting total solids of camel milk with camel milk protein powders to obtain a firm texture has not been investigated

1.7.4 Fermented yoghurt

1.7.4. 1 Aims

To develop a fermented spoonable camel milk yoghurt

1.7.4.2 Novelty

A spoonable fermented camel milk yoghurt low in E number additives has not been reported

1.7.5 Camel milk insulin

1.7.5.1 Aims

- Determination of the insulin concentration and insulin-like components by immunochemical methods (ELISA) using anti-insulin antibodies from human and bovine origin;
- Localisation of insulin in the whey or casein fraction of camel milk by immunochemical methods;
- Determination of the molecular weight of camel milk insulin by immune blotting;
- Measurement of insulin biological activity in camel milk by an *in vitro* method.

1.7.5.2 Novelty of this study

To determine the physical presence of insulin in camel milk and measure its concentration;

- The only reported way to detect the physical presence of insulin in milk is based on radioimmunoassay. In this study, ELISA will be used with bovine insulin and iso-insulin antibodies for the first time.
- The molecular weight of insulin will be done by immune precipitation be, SDS PAGE and Western Blot analysis. This information could not be found in peer-reviewed publications.
- To measure insulin biological activity *in vitro with* human hepatic cell line, which has not been reported in the literature.

CHAPTER 2

COMPARISON OF THE EFFECT OF HEAT TREATMENT ON CAMEL AND BOVINE SKIMMED MILK AND WHEY

2.1 Introduction

Camel milk plays a central role in the food supply of Eastern African countries (e.g. Kenya, Somalia, Sudan, and Ethiopia), which contain the majority of the world's camel population. Raw, or traditionally fermented, camel milk has become increasingly commercialised and consumed in urban areas. This has led to an increased interest in the processing of camel milk for the urban market, with skimmed camel milk and whey powders soon to be commercialised, in a similar fashion to the widely available bovine skimmed milk and whey protein powders. However, little information is currently available concerning the effect of processing methods (e.g. thermal treatment) on camel milk fractions. This is of particular interest as these protein products will be manufactured, and utilised for products, such as yoghurt, a popular food product with many health attributes.

2.1.2 Composition of proteins in camel milk versus bovine milk

The composition of camel and bovine milk has been described in Table 2, Chapter 1. The composition of proteins in camel and bovine milk is outlined in Table 9 Chapter 2.

Table 9 Comparison of approximate protein compositions between liquid camel and bovine milk. (Information obtained from Ali Al-Alawi and Laleye, 2015 and El- Hatmi et al., 2014)

Protein	Bovine milk %	% in liquid	% of total protein	Camel milk %	% in liquid	% of total proteins
α casein	1.5	Casein 2.85	Casein 83.5	0.69	Casein 1.45	Casein 37
β casein	1.00			0.69		
κ casein	0.30			0.07		
αLactalbumin	0.13	Whey 0.56	Whey 16.4	0.10	Whey 2.43	Whey 63
β lactoglobulin	0.30			0.03		
serum albumin	0.08			1.12		
IgG	0.05			1.15		
Total protein	3.41		100	3.88		100

Table 9 demonstrates that the ratio of casein and whey in bovine milk is 83.5% and 16.4%, respectively, whereas for camel milk the ratio is 37% for casein and 63% for whey protein (El-Hatmi et al., 2007).

Camel milk contains lower casein fractions in comparison to bovine milk, and the main component of camel whey is camel serum albumin (CSA), whereas in bovine whey it is β- lactoglobulin (El-Hatmi et al., 2007).

2.1.2.1 Casein

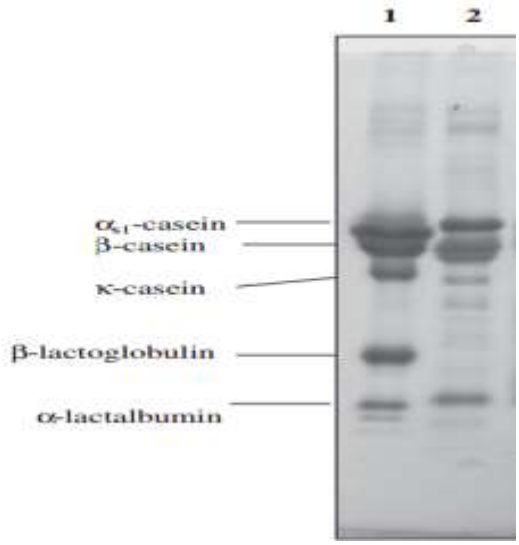

Figure 5 SDS-PAGE electrophoretogram of lane 1: Bovine milk. Lane 2: Camel Milk (Hinz et al., 2012)

Figure 5 demonstrates the difference in molecule weight between camel and bovine milk, with camel and bovine lanes (1 and 2) have three casein's bands. However, they differ in size and intensity (Hinz et al., 2012).

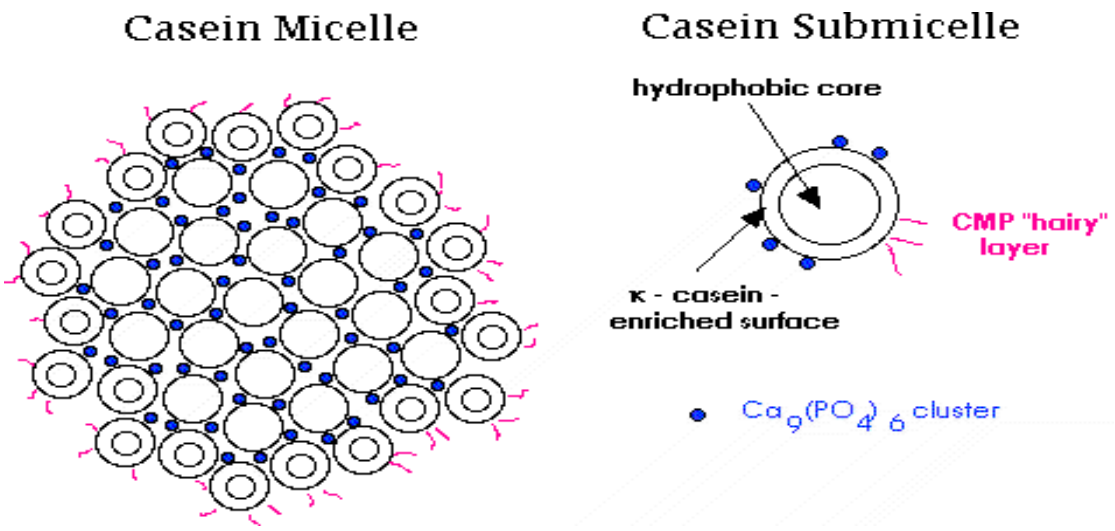

Figure 6 Structure of casein micelles in bovine milk (Euston, 2010)

"Bovine casein micelles are spherical particles, which do not appear smooth in electron micrographs, but have been likened to the surface texture of a raspberry. A substructure can be observed in unstained thin sections of casein micelles, with regions of greater electron density dispersed evenly in a less dense matrix. When bovine casein micelles are dissociated, spherical particles are observed with a size similar to the scale of the substructure" (Euston, 2010).

Caseins are phosphoproteins containing approximately 80% of the total protein content of milk protein. Casein is formed from a large number of components, the main types being as follows: αs_1- casein, αs_2-casein, β-casein, and κ-casein. Caseins are not heat sensitive, and it is only temperatures up to, or above, 120ºC that cause the casein to gradually become insoluble. However, they are sensitive to pH and will precipitate at its isoelectric pH (Slattery, 1976).

Camel casein contains less α- casein, β-casein and κ-casein than bovine casein Table 9 The most significant difference is that of κ-casein in camel casein is 0.07% compared to 0.3% for bovine casein , which could be responsible for the lower acid coagulation properties

2.1.1.2 Whey

Camel whey is deficient in β-lactoglubulin), a major component of bovine whey, whereas CSA is the major component of camel milk (El-Hatmi et al., 2007, Merin et al.,

2001; Sawyer, 2013; Yang et al., 2014. Whey proteins are acid stable in a non-denatured form, and do not precipitate unless denatured.

2.1.3 Effects of the acidification of milk

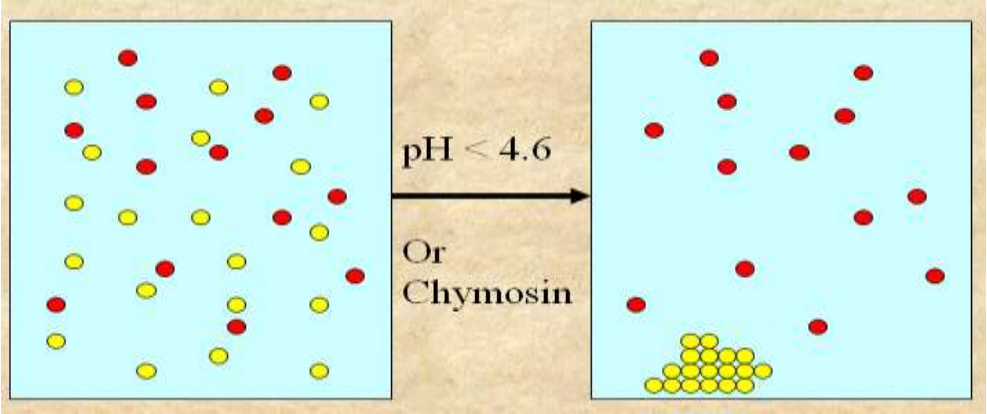

Figure 7 Precipitation of milk casein protein from camel milk (S. Euston, 2010)

If an acid is added to bovine milk, the negative charges on the outer surface of the casein micelles are neutralised through means of the protonation of the phosphate groups. When acidified, the casein micelles are destabilised or aggregate, because the electrical charge that decreased to the isoelectric point (i.e. pH at which there is no net charge due to the presence of an equal number of positive and negative charges). The casein micelles disintegrate, while the casein (i.e. the neutral protein) precipitate because it is no longer polar, while the calcium ions remain in solution (Spurlock, 2015).

The iso electric point of camel milk is pH 4.3. Wangeoh et al. (1998) precipitated camel casein at a different pH, between 3.55 and 5.30 at 20°C, concluding that the most effective separation of casein and whey proteins of camel milk took place at pH 4.3. These results reveal the fact that the precipitation of casein in camel milk cannot be performed in the same manner as in bovine milk, in which casein is precipitated by the acidification of milk at 20°C to pH 4.6. This low pI of camel milk casein has implications on the determination of casein in camel milk. In the literature, casein in camel milk has been precipitated at the same pH as casein in bovine milk. Wangoh et al. (1998) report that if camel casein is precipitated at pH 4.6, a proportion of casein will remain in the whey, leading to a subsequent underestimation of the casein content

2.1.4 Effect of heat treatment on milk properties

2.1.4.1 Heat treatment of milk

Milk subjected to heat treatment to ensure microbiological safety before retail and consumption. There are three types of heat treatment: (1) low temperature long duration (LDLT) pasteurisation (15 seconds at 56°C); (2) high temperature short duration (HDST) pasteurisation (72°C); and (3) ultra-high temperature (UHT) treatment (5 seconds at 140°C). (Shimamura and Ukeda, 2012; Lacroix et al., 2008).

Vasbinder and de Kruif (2003) noted the ways in which heat treatment of bovine milk affects the interaction between the whey and the casein. Heat treatment denatures whey proteins, leading to a mixture of whey protein aggregates and whey protein-coated casein micelles. During the heat treatment of bovine milk, β-lactoglobulin covalently interacts with κ-casein on the exterior of the casein micelles, through means of disulphide-linked bonds. Whey protein also denatures and forms disulphide bonds with surrounding whey proteins forming whey protein aggregates. Heated milk is thus a mixture of native and denatured whey proteins, which occur as whey protein aggregates, or as a coating present on casein micelles.

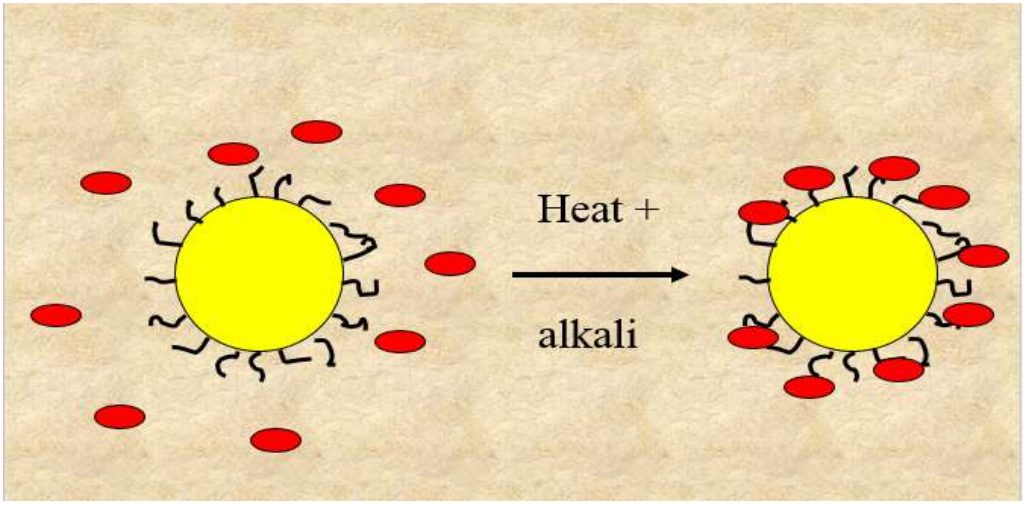

Figure 8 β-lactoglobulin/κ-casein complex (S. Euston, 2010)

2.1.4.2. Denaturation of whey protein

2.1.4.2.1 Molecular dynamics

The thermally induced denaturation of bovine whey proteins occurs in two phases: (1) the unfolding of the native state and (2) aggregation with the unfolded molecule (Fox, 2003). During the first stage, the secondary and tertiary structure of the proteins become

disrupted, leading to the molecules adopting a different conformational state through: (1) the breaking of hydrogen bonds; (2) the uncoiling of polypeptide chains; (3) the exposing of reactive groups. The exposure of hydrophobic and SH groups (which remain buried in the protein core at ambient temperatures) is frequently referred to as the 'hydrophobic effect'. This is associated with a decrease in the proportion of α-helices, and an increase in the proportion of hydrophobic β-sheets. The molten globule state has been described as a partially unfolded conformation that is distinct from both the native, and the fully denatured, states. The molten globules form soluble aggregates primarily through hydrophobic interactions mediated by the exposed areas on the ß-sheet, resulting in soluble aggregates. The first denaturation stage does not involve disulphide bond formation (Fox, 2003).

Further heating leads to the following stage of denaturation, resulting in additional denaturation of the soluble aggregates and both disulphide bond formation and gelation (Fox, 2003).

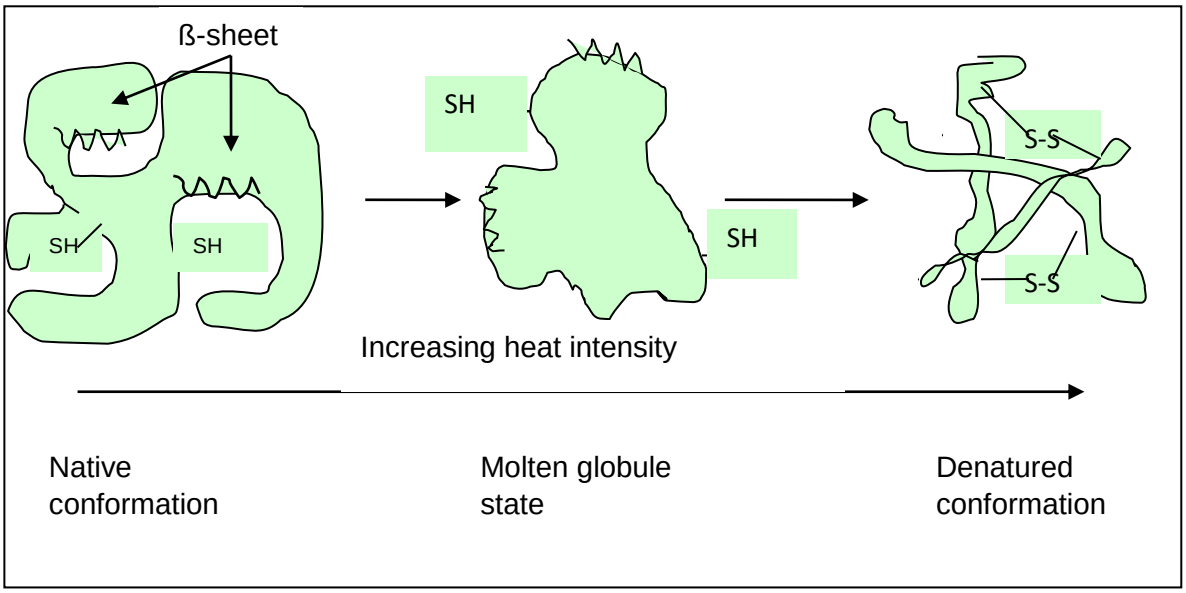

Figure 9 Different conformations of globular proteins during heat-induced denaturation (L. Campbell., 2010)

The quantity of SH groups reflects the number of disulphide bonds that form following heat treatment, which is a key step in the gelation process of the protein.

Approximately 96% of the total number of SH groups (excluding S-S bonds) associated with 100g of bovine whey protein (536 µmol) are associated with β-lactoglobulin (Apenten, 2005). The total SH in 1g bovine milk is 5.54 umol (Apenten 2005).

The other whey proteins, (i.e. α-lactalbumin) lack free thiol group to serve as the starting point for a covalent aggregation reaction. As a result, pure α-lactoalbumin will not form gels upon denaturation and acidification. Although bovine serum albumin constitutes only 0.08% of bovine whey, it accounts for 2–4% of the total number of SH groups (Owusu-Apenten, 2005).

In camel whey, the major protein is camel serum albumin, followed by IgG, and there is no β-lactoglobulin Table 9 presents by (El Hatmi et al., 2015).

2.1.4.2.2 Principle of measurement of free and total SH groups

2.1.4.2.2.1 Free SH

As heating progresses, protein molecules unfold, disrupting the secondary and tertiary structures, and thus exposing the sulfhydryl (or thiol or SH) groups. Free SH (FSH), also known as reactive SH, comprise the SH group located on the surface of the protein molecules. Due to the fact that FSH is a measure of both native and heat-exposed SH groups, it can be considered as a measure of protein denaturation, and therefore an indicator of the functional performance of the denatured protein. The FSH indicator is measured by reacting 5,5' dithiobis (2-nitrobenzoic acid) (DTNB) with the SH groups to form dianion 2-nitro-5-thiobenzoic acid (TNB^{2-}). TNB^{2-} is yellow, with the intensity of the colour measured by colorimetry at 420nm wavelength and quantified as a measurement of SH groups (Owusu-Apenten, 2005).

2.1.4.2.2.2 Total SH

Not all proteins are completed unfolded during heat treatment. In order to quantify the degree of denaturation, it is essential to measure all the SH groups of the protein, including those residing inside the unfolded structure. Total SH (TSH) is measured by the addition of SDS and urea to completely unfold the protein (Owusu-Apenten, 2005).

2.1.3.3 Maillard reaction (Glycation)

The Maillard reaction was first identified by French chemist Louis Maillard, and was described in more detail in 1953 by Hodge (Martins et al., 2000). It plays an important role in both the appearance and taste of foods, and has formed a central challenge in the development of desirable aromas, tastes, and colours, particularly in relation to the roasting of coffee and cocoa beans, the baking of bread and cakes, the toasting of cereals, and the cooking of meat (Nursten, 1981; Ames, 1998). A wide range of reaction products are formed during the Maillard reaction, which has the potential to: (1) reduce

the nutritional value of foods (i.e. by decreasing their digestibility and increasing the formation of toxic and mutagenic compounds); (2) increase nutritional value (i.e. by increasing the formation of antioxidants) (Martins et al., 2000).

The Maillard reaction is a non-enzymatic glycation reaction between the amino group of a protein and the carbonyl group of a reducing sugar. It generally takes place during food processing or storage. The chemistry underlying the Maillard reaction is highly complex, encompassing multiple reactions (Shimamura and Ukeda, 2012).

The amino acid lysine is most likely to undergo glycation reactions, because its primary amino group is particularly prone to reactions (Labuza, 2005). In milk, lactose (i.e. the main carbohydrate) reacts with the free amino acid side chains of milk proteins (primarily ε-amino group of the lysine residue) during the early, intermediate, and advanced stages of the Maillard reaction, to form a variety of reaction products. The first reaction generates an Amadori product, which progresses down the 3-deoxyosone or 1-deoxyosone pathway, depending on the reaction pH. The 4-deoxyosone can also be formed in the Maillard reaction of disaccharides, e.g. lactose. Finally, the Maillard reaction results in the formation of browning compounds called melanoidins (Shimamura and Ukeda, 2012; Taheri-Kafrani et al., 2009; Thi Tuyet Mai, 2012; Martins et al., 2000; Van Boekel, 1998).

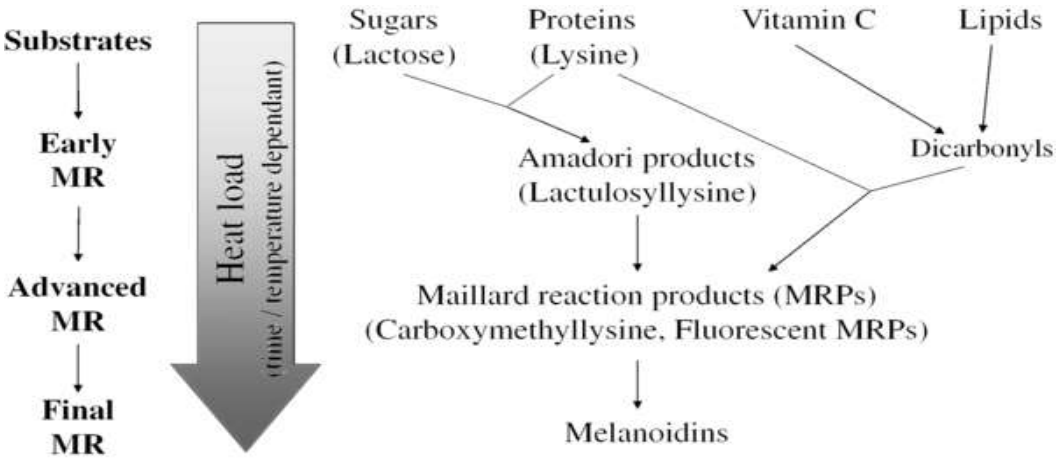

Figure 10 Maillard reaction stages in milk with increasing temperatures of heat treatment (Roux et al., 2009)

The Maillard reaction occurs in milk during all types of heat treatment (Shimamura and Ukeda, 2012; Lacroix et al., 2008). The reactions of lactose and milk proteins have been frequently investigated, and the formations of various Maillard reaction products in milk during heat treatment have been demonstrated. During high temperatures, the

nucleophilic amino acid residues of milk protein (e.g. lysine and cysteine) become highly active and can, in the present of lactose (which is reactive due to its carbonyl group), form intra-molecular bonds. Heat induces polymers at a temperature of 80°C, and polymerisation may result from many forms of crosslinking, including disulfide bonds, i.e. crosslinking arising from the Maillard reaction (Fox, 2003).

The initial stage of the Malliard reaction produces an unstable glycosylamine, which can be rearranged, and/or break down into a number of different products, depending on the reaction conditions (e.g. temperature and pH) as well as the type of sugar and amino acid/protein employed (Martins et al., 2000).

The Maillard reaction has various effects on the bioavailability, solubility, foaming properties, emulsifying properties, and heating stability of milk proteins. The glycated β-lactoglobulin is more stable during heat treatment at an acidic pH (Fox, 2003). Moreover, the glycation of β-lactoglobulin may improve its foaming and emulsifying properties. These results suggest that the Maillard reaction is beneficial in the modifying of the properties of milk proteins (Hidalgo and Zamora, 2005; Liu et al., 2003).

2.1.5 Functional properties of milk proteins

The important functional properties of whey protein products (including water binding, emulsification, foaming, whipping, and gelation) depend on protein solubility. Heat treatment causes denaturation of whey milk and results in considerable alteration of the protein's functional properties, e.g. viscosity and emulsification (Morr and Ha, 1993). Fox (2003) reports that heat treatment can considerably alter functionality, and even whey proteins with identical ingredients can contain very different functional properties, e.g. improvements in the viscosity and texture of the product.

2.1.5.1 Effect of solubility

Protein solubility is defined as the ability to associate with water, and is a function of a number of factors, including solvent, pH, temperature, and ionic strength of the protein. The solubility profile is frequently the first property assessed at each stage of preparing a protein ingredient, as it assesses the functional application of proteins to optimise protein extraction and processing procedures (Rupnow, 1992). Protein insolubility reflects the extent to which protein aggregation and denaturation occurs, and influences its emulsification, hydration, foaming, and gelling properties. Ahmed (2013) is of the opinion that the changes in the solubility and foaming properties of whey proteins with

mild heat treatments up to 60°C have been demonstrated to be reversible, while the functional properties of solubility are affected irreversibly at higher heating temperatures.

2.1.5.2 Emulsifying properties

The emulsifying properties of a protein define its ability to stabilise an emulsion, an important factor in the development of food products, including soups, salad dressings, mayonnaise, and cakes (Jackman et al., 1989). These properties are generally discussed in terms of emulsifying activity, capacity, and stability (Pearce and Kinsella, 1978). Emulsions consist of one, or more, lipophilic liquids and one, or more, polar hydrophilic liquids (Al-Malah et al., 2000). Proteins are effective surface-active agents, because they lower the interfacial tension between the hydrophobic and hydrophilic components in foods.

Proteins in the emulsion system have a stabilising effect by forming a protective barrier around fat droplets, thus preventing coalescence of the emulsion (Zayas, 1997). The two basic forms of emulsions consist of dispersion of an oil (or lipophilic) phase in a watery (or hydrophilic) phase. Water and oil are the two most commonly used liquids for the production of food emulsions globally, and are present as water-in-oil (W/O) emulsions and oil-in-water (O/W) emulsions. (Schubert et al.,2006). The capacity of protein to stabilise emulsions is related to the interfacial area on the coating of the protein. Emulsion stability is defined as the capability of emulsion droplets to remain dispersed without creaming, flocculating, or coalescing (Zayas, 1997).

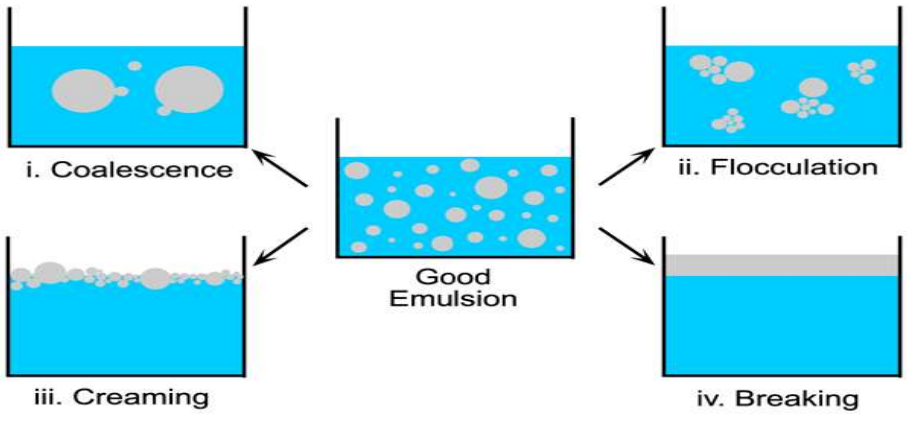

Figure 11 Emulsion stability is defined as the capability of emulsion droplets to remain dispersed without creaming, flocculating, coalescing, or breaking (Zayas, 1997).

2.1.5.3 Emulsification properties related to an emulsion viscosity

Due to the fact that proteins lower the interfacial tension between hydrophobic and hydrophilic components in foods, this gives the potential to reduce the size of the oil droplets in emulsions, which could lead to increased viscosity. The ability of protein to reduce oil droplet size, and thus increase emulsion viscosity, is a desired functional property. Small oil droplet size leads to higher levels of viscosity, as a higher number of smaller particles results in an increase in both particle-particle interactions and resistance to flow (Fletcher, 2015).

2.1.5.4 Water binding ability of protein in an emulsion

The water absorption capacity of a protein defines its ability to absorb and retain water, which forms an important component of many foods, e.g. dough and comminute meat products (Kinsella and Melachouris, 1976; Rupnow, 1992). Water absorption capacity is commonly measured by the amount of water retained by a protein powder sample after blending it with water, then and removing excess water through centrifugation (Zayas, 1997). Chou and Morr (1979) have demonstrated that water absorption capacity depends on a number of factors, e.g. the hydrophilic–hydrophobic balance of amino acids in the protein molecule, and the carbohydrate fractions associated with the protein.

2.2. Materials and Methods

2.2.1 Materials

2.2.1.1 Raw food materials

- Raw camel milk was obtained from Bradford, UK (Kamelus, the home of 'White Gold').
- This source was discontinued and the decision was made to obtain pasteurised camel milk from Saudi Arabia *(al-turath al- Saudi).* This was then exported in frozen form to Edinburgh, in order to enable the experiments to continue.
- Bovine pasteurised skimmed milk was bought from the local supermarket (ASDA Fresh Skimmed Milk).

2.2.1.1.2 Chemicals

The following was obtained from Sigma aldrich

- Bovine serum albumin, cat no: A2153.
- Bradford reagent, cat no: B6916-500ML.
- TNBS (2, 4, 6-trinitrobenzenesulfonic acid), cat no: 763586.
- 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB), cat no: D218200.
- Acetic acid, cat no: A6283.
- Hexane, cat no: 296090.
- Lactose Assay Kit, cat no: MAK017-1KT.
- Sodium hydroxide, cat no: S8045.
- Hydrochloride, cat no: 857645.
- Lactose, cat no: 17814.

2.2 Methods

2.2.1 Preparation of bovine and camel milk fractions

Both the bovine and camel milk were skimmed (i.e. defatted) by centrifugation of the samples at 5000 rpm for 20 minutes, in 50ml centrifuge tubes placed in a Denley BS400 (Model 65400) centrifuge. Samples were cooled to 4°C, following which any solid fat was removed from the top of the milk by means of a metal spatula.

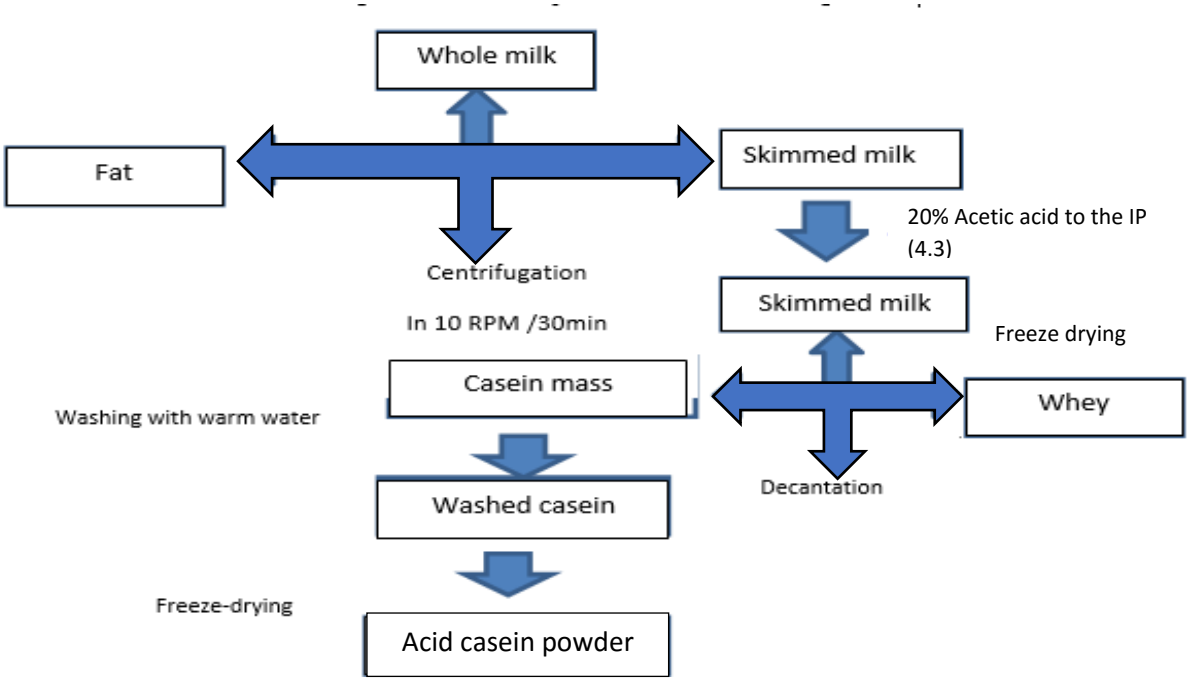

Figure 12 Flow diagram for whey and casein preparation (Evapodry.com, 2014)

2.2.2 Preparation of whey and casein fractions

The methods employed were those described by Salami et al. (2011). For the preparation of whey fractions, the casein was precipitated at pH 4.6 for bovine milk and 4.3 for camel milk using 20% (v/v) acetic acid, followed by centrifugation at 3000 rpm for 20 minutes. The supernatant, which constituted the whey, was decanted into containers. Casein was washed 3 times with distilled water then re-solubilised by adjusting the pH to 7, through the use of sodium hydroxide NaOH 20%. The casein solution was stirred for 30 minutes until fully dissolved

2.2.3 Proximate analysis of milk powder

Total ash

The ash content was determined by placing 4 g of dried sample in a muffle furnace and, after ignition, maintaining a temperature of 550°C (as described in Aaccnet.org, 2000: method No. 08-01).

% of total ash: Sample weight after muffle furnace /Sample weight before muffle furnace *100

Total protein

The nitrogen was determined by a Kjeldahl procedure, as described by Markham (1942). A factor of 6.38 was used to convert nitrogen to crude protein in the following equation.

Protein %:(Nitrogen) N x 6.38/sample * 100

Total fat

The method employed was that of solvent extraction using a Soxhlet extraction, as described in method No. 30-10 (Aaccnet.org, 2000). Samples of 2g of dried milk powder were placed in a thimble, and positioned in the extraction tube of the Soxhlet apparatus. Approximately 250ml of hexane were added in the 500ml bottom flask of the apparatus, and then connected to the Soxhlet apparatus. The fat was extracted by running hexane over the sample at the rate of between 3 and 4 drops per second, for duration of approximately 5 hours. The contents of the flask were transferred to a pre-weighed petri dish and dried on a hot plate for 10 minutes, at a temperature of between 40°C and 50ºC. The petri dish was cooled in a desiccator and then weighed. The percentage of fat was calculated according to the following formula:

Fat%

Crude fat / Weight of sample * 100

Lactose

A standard curve was prepared, as follows**:** 5ml lactose monohydrate solutions in the concentration ranges 0.55 x 10^{3}, 1.11 x 10^{-3}, 1.66 x 10^{-3}, and 2.22 x 10^{-3} molar were prepared according to the $PdCl_2$ method (Petrushevska-Tozi et al., 1997). The sedimentation of substances in samples of milk was achieved using weak alkaline Fehling I Solution. The pH of the serum obtained was 6. A 5ml milk serum sample solution and 10ml of reagent 2 (5ml of 5.65x10^{-2} molar $PdCl_2$), was heated at 70°C for 50 minutes in a water bath. The mix was transferred into a volumetric flask and diluted to 100ml with water. 5ml of transparent liquid was transferred into a 25ml volumetric flask, and 5ml of KI solution 3 (6 x 10^{-1} M) and 1ml of 5 x 10^{-1} molar HCl solution were added and diluted to 25ml with water. After 5 minutes, the absorbance was measured at a ƛ= 410nm wavelength. The lactose content was calculated according to the standard curve (Petrushevska-Tozi et al., 1997). A further method has also applied to measure the lactose in the milk. This was a lactose assay kit provided by Sigma (cat no MAK017-1KT), and which gave the same results as the method described above.

2.2.4 Heat treatment of skimmed milk and whey

Skimmed milk and whey samples were placed in quantities of 1 litre each in separate 2 litre glass bottle with a cap. The bottles were placed in a shaking water bath set 5 ºC higher than the target temperature, e.g. for a 60 ºC target temperature, the water bath was set at 65ºC. The samples were heated to 60, 70, 80 and 90 ºC. The temperature of the samples was measured intermittently with a thermometer, after which the lid was replaced immediately. Each bottled sample was kept at its target temperature for 5 minutes and then put on ice to cool. Part of each sample was set aside, in order to measure solubility, turbidity, free and total SH groups and of glycation. A number of samples were not subjected to heat treatment, to enable them to be used as a control.

2.2.5 Freeze drying of samples

All heat-treated samples were freeze dried, as well as the non-heated samples (i.e. the control) and casein.

2.2.5.1 Measurement of protein physicochemical properties of heat treated liquid skimmed milk and whey

2.2.5.1.1 Turbidity

Turbidity is a principal physical characteristic of water, and is an expression of the optical property that causes light to be scattered and absorbed by particles and molecules through a water sample, rather than transmitted in straight lines. It is caused by suspended matter, or impurities, that interfere with the clarity of the water. A sample of 1ml was transferred to a glass cuvette (3ml). Turbidity measurements were carried out at wavelength of 600nm, as described by Aschaffenburg, 1950 and Humbert et al., 2006, using a Genesys 6 spectrophotometer (Thermospectronic, USA). Colour was visually evaluated and photographed. Each heated sample, turbidity was then compared to a non-heated sample.

2.2.5.1.2 Measurement of soluble protein

The ready-to-use Bradford reagent is based on an acidic coomassie-dye reagent, which changes colour from brown to blue, depending on the concentration of protein in the sample. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs (Bradford, 1976).

A 1% bovine serum albumin (BSA) solution was used to make a standard curve, ranging from 10 to 30µl of the BSA dilution. Milk samples at different dilutions were prepared with the Bradford reagent. Samples were mixed with a range of milk (1, 5, 10 and 15µl) and 1ml of Bradford reagent. The samples were then left them at room temperature for 3 minutes before measurement took place of the absorbance at wavelength 595nm. Results were obtained by plotting sample values against the BSA standard curve.

2.2.5.1.3 Protein solubility

Heat-treated liquid whey and milk samples were centrifuged at 5000 rpm for 20 minutes. The soluble protein content in supernatant was determined by the Bradford procedure, as described in section 2.5.2. Each heated sample was compared to an identical sample that had not been heated, in order to calculate the degree of denaturation.

2.2.5.1.4 Free and total SH group

Free SH groups (FSH) were determined by the addition of 300 µl of the sample to 5ml Tris-glycine buffer (0.086 M Tris, 0.09 M glycine, 0.004M Na2EDTA, pH 8), followed by an addition of 200 µl 0.02 M DTNB. The solution was vortexed and left to react at RT for 15 minutes, before recording the absorbance at 412nm using Genesys 6 spectrophotometer (Thermospectronic, USA). The blank for each measurement was the sample prepared using the described procedure, but with the omission of the DTNB. For determination of total SH groups (SHT), 300 µl of each sample was added to 5ml Tris-glycine buffer pH 8 (containing 6 M urea and 0.5% SDS), 200µl of 0.02M DTNB buffer was then added, and absorbance at 412 nm was measured, as described for FSH groups. The percentage denaturation was calculated as a native sample value - heated sample value.

SH measurement calculation (mol SH/litre) in milk

This was undertaken according to the formula outlined by Owusu-Apenten (2005):

Δ A 412/ E 412 * (v-ml)/1000 * ϕ * 1000/(vsml)

E 412: 13600

v-ml: total volume of sample in cuvette

ϕ: fraction of assay volume in cuvette

vs- ml: original volume of food sample

2.2.5.1.5 Glycation degree (i.e. measurement of free amino groups (NH-groups)

This was undertaken according to the method published by Fields (1972). TNBS (2,4,6-trinitrobenzene sulfonic acid) component reacts readily with primary amino groups of amino acids in aqueous format at pH 8, to form yellow adducts The reaction of TNBS with primary amines generates a highly chromogenic product that can be readily measured at a wavelength of 420 nm.

Glycation buffer: 0.1 M sodium tetraborate solution (pH 9.3). Colour reagent: 0.03M TNBS (2, 4, 6-trinitrobenzenesulfonic acid).

100 µl of the milk sample was added into 1ml glycation-buffer in a cuvette. This was placed into a spectrophotometer (i.e. Genesys 6 spectrophotometer [Thermospectronic, USA]) set at an absorbance wavelength of 420 nm and blanked. 5 ul colour reagent was added, mixed and allowed to react for 60 seconds. Absorbance was read again at 420 nm.

2.2.6 Measurement of particle size of casein fraction

Particle size was measured with a Zetasizer Nano Z particle size analyser by dynamic light scattering. Casein powder was adjusted to pH 7, with addition of 1 molar Sodium hydroxide. The powders were dissolved at g 4% protein percentage in water with adjusted pH 7±0.5 (Elofsson et al., 1996) and vortexed to provide homogeneous casein.

2.2.7 Measurement of protein functionality in emulsion

2.2.7.1 Preparation of emulsion

A 3.75% skimmed milk or whey powder solution was emulsified with 60ml oil (1.5g skimmed milk powder + 40ml water + 60ml oil) for 1 minute with an Ultraturrax benchtop homogeniser at 20,000 rpm. It was then homogenised for another 2 minutes at 30,000 rpm.

2.2.7.2 Measurement of the oil droplet size of emulsions

The average emulsion oil droplet size distribution (i.e. $D_{3,2}$ surface weighed mean) was measured by laser light diffraction, using Mastersizer 2000 equipment (Malvern instruments Ltd Malvern, UK) at a refractive index of 1.470, Emulsion droplet size was established by dropping the emulsion slowly until the laser obscuration index reached approximately 5%.

2.2.7.3 Measurement of viscosity of emulsions

A Bohlin Gemini rheometer (Malvern Instruments Limited, Worcestershire, UK) was used to measure the viscosity of samples at a controlled shear rate (0.10–100 /s). A cone and plane geometry, with a cone angle of 4° (C4/40), and a cone diameter of 40 mm, was used, and data recorded for samples in a duplicate at room temperature.

2.2.7.4 Emulsion's water separation

The emulsion was prepared in a calibrated cylinder in order to clearly measure the water separation. After 24 hours' incubation in 4ºC, water released from the emulsion was measured as follows: Water volume on top of the emulsion after 24hrs (ml) / volume of sample before incubation*100.

2.3 Results and Discussion

2.3.1 Properties of liquid skimmed milk and whey (not freeze dried)

2.3.1.1 Protein concentration as measured by Kjeldahl

Table 10 Protein concentration of liquid camel and bovine milk and whey fractions

Heat treatment °C	Protein concentration%			
	Camel whey	Bovine whey	Camel milk	Bovine milk
20	2.4 ±0.12	0.52 ±0.21	3.6 ±0.07	3.54 ±0.49

Liquid camel whey contains a 4.6 times higher protein content than bovine whey (i.e. 2.4% versus 0.52%). This corresponds to published values for camel whey (El Hatmi et al., 2014) and bovine whey. Moreover, the protein concentration in camel milk is higher than in bovine milk (i.e. 3.6% versus 3.5%) This correspond to published values for camel milk and bovine milk (Al-Alawi and Laleye, 2015; Suleiman et al., 2014)

2.3.1.2 Colour of whey

The colour of milk is perceived by consumers to be indicative of its purity and richness. The fact that it appears to be white is due to the scattering of reflected light by the inherent ultramicroscopic particles, fat globules, colloidal casein micelles, and calcium phosphate. The intensity of this whiteness is directly proportional to the size and number of particles in suspension. Bovine milk contains the pigments carotene and xanthophyll, which tend to give a golden yellow colour to the milk fat.

Figure 13 Colour of bovine whey (1) and camel whey (2) samples

Sample 1 consists of bovine whey, and sample 2 consists of camel whey, after acid precipitation of the casein. The colour of camel whey is clearly whiter than that of bovine whey. A possible reason could be the low concentration of riboflavin in camel milk, as compared to bovine milk. This has been reported by Ramet (2001) and El-Agamy (2006) as 41 and 150 µg/100 g, respectively.

2.3.1.3 Particle sizes of casein

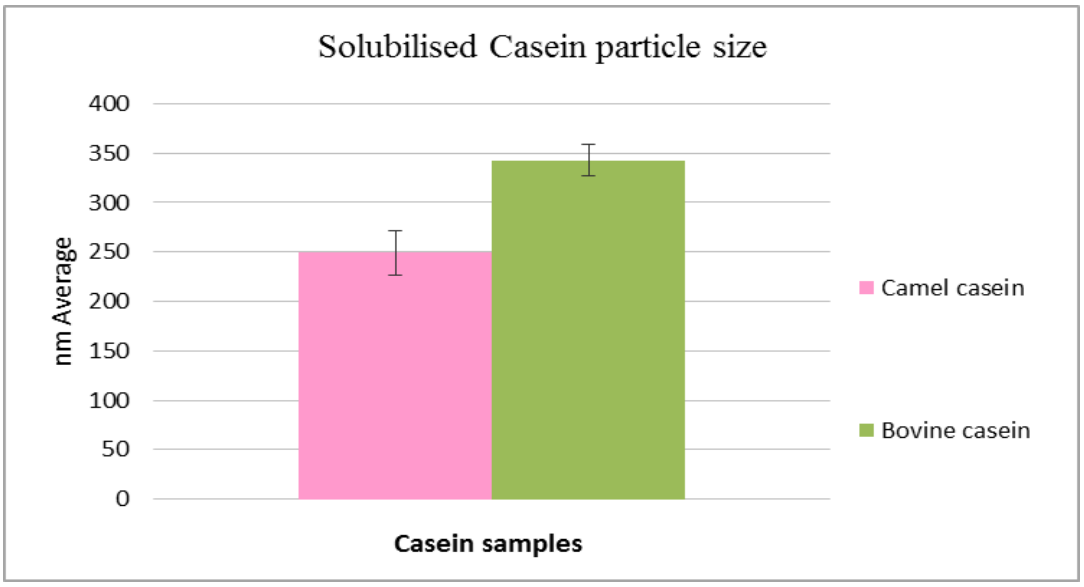

Figure 14 Average particle sizes of solubilised bovine and camel casein fractions, as measured by the zeta sizer. All samples were native (i.e. non-heat treatment)

Camel solubilised casein revealed lower particle sizes compared to bovine casein. This confirms the results of Farah and Ruegg (1989), Ali and Robinson (1985) and Ono et al., 1990), who reported an average micelle diameter of approximately 280 nm for camel milk casein, compared to the bovine casein with an average micelle diameter of approximately 300 nm.

2.3.2 Physicochemical properties of heat treated liquid bovine and camel skimmed milk and whey

All samples were undertaken in triplicate, and statistical analysis was applied with Excel. Standard deviation was employed to measure the amount of variation of set values, however, the standard deviation value for some data was too small to be displayed on the chart. The standard deviation values consisted of ≤ 0.05 for Turbidity, solubility, glycations and SH groups.

2.3.2.1 Turbidity

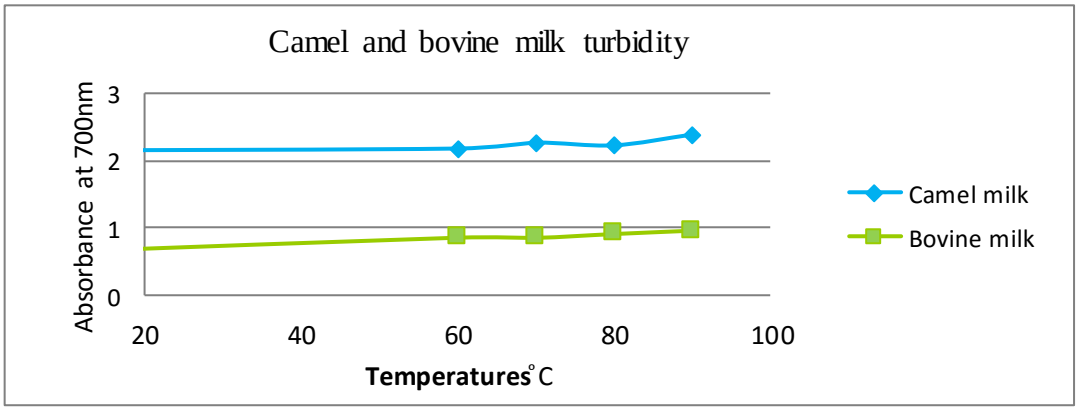

Figure 15 Turbidity of skimmed camel and bovine milk (before freeze-drying)

Figure 15 Subjected to different heat treatments at neutral pH. Figure 15 indicates that camel milk possesses higher turbidity than bovine milk; due to the fat content in camel skimmed milk being doubles that of bovine skimmed milk, as determined by the proximate analysis of powders

Table 11. The fat was still present in camel skimmed milk even after the defatting process. Farah and Rüegg (1991) reported that camel milk is difficult to cream, due both to the small size of the fat globules.

The turbidity of bovine and camel milk increased with an increase in temperature. Anema and Klostermeyer (1997) report that the dissociation of κ-casein in bovine milk increases linearly with the increase of the heating temperature (10 -90ºC). Changes in casein micelle size have been related to the levels of denatured whey proteins associated with the casein micelles (Anema and Li, 2003). These phenomena cause an increase in the turbidity of heat-treated skimmed milk.

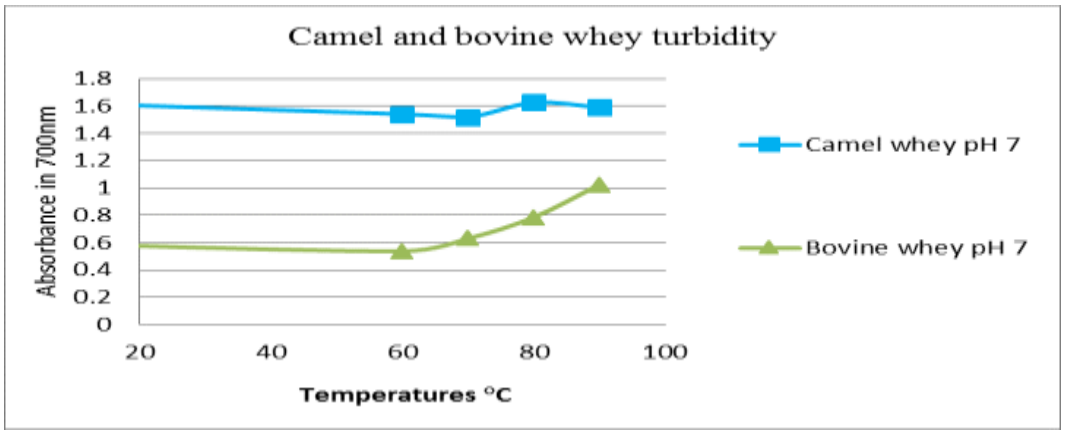

Figure 16 Turbidity of camel and bovine milk whey subjected to different heat treatments at pH 7

Figure 16 reveals a higher turbidity for camel whey, as compared to bovine whey. This could be due to the higher protein concentration in camel whey, along with the higher fat content demonstrated in **Table 12**. Bovine whey turbidity increases more with increasing heat treatment in comparison to camel whey, indicating higher heat stability for camel whey.

2.3.2.2 Milk protein solubility

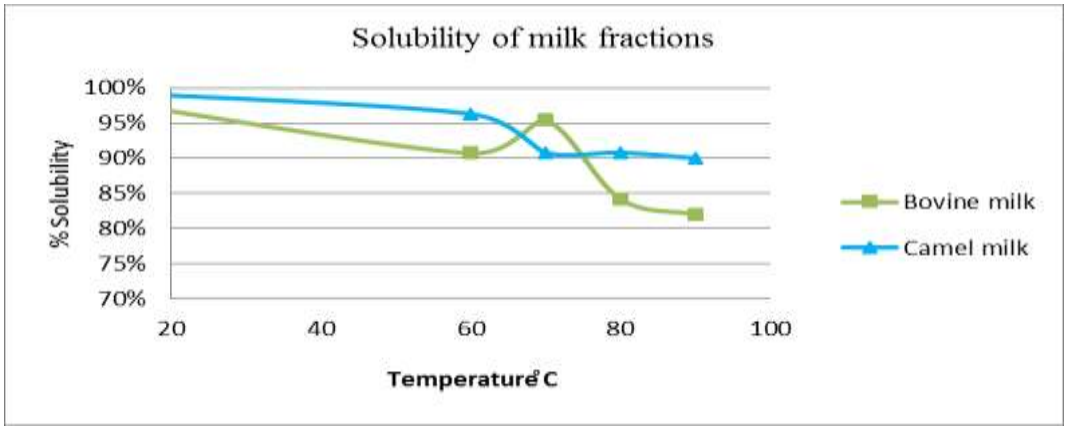

Figure 17 Protein solubility after heat treatment of skimmed milk at neutral pH.

Figure 17 demonstrates that camel milk possesses a higher solubility than bovine milk at high temperature

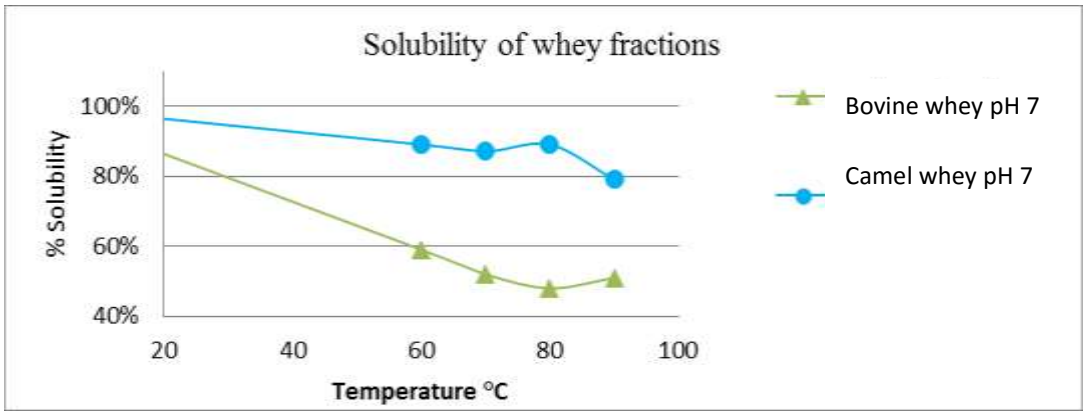

Figure 18 Protein solubility of liquid whey after heat treatment and neutral pH.

The results in Figure 18 reveal that bovine whey at pH 7 undergoes a greater decrease in solubility than camel whey at the same pH under heat treatment. Camel whey proved to be moderately heat stable, confirming the results of Laleye et al. (2008). This difference could be due to the absence of β-lactoglobulin in camel whey, which increases its stability in relation to heat (Ryan, et al. 2013). The decrease in the solubility of bovine whey corresponds to increasing in turbidity at neutral pH .This demonstrates the formation of insoluble aggregates in bovine whey, whereas camel whey remains relatively soluble.

2.3.2.3 Glycation degree

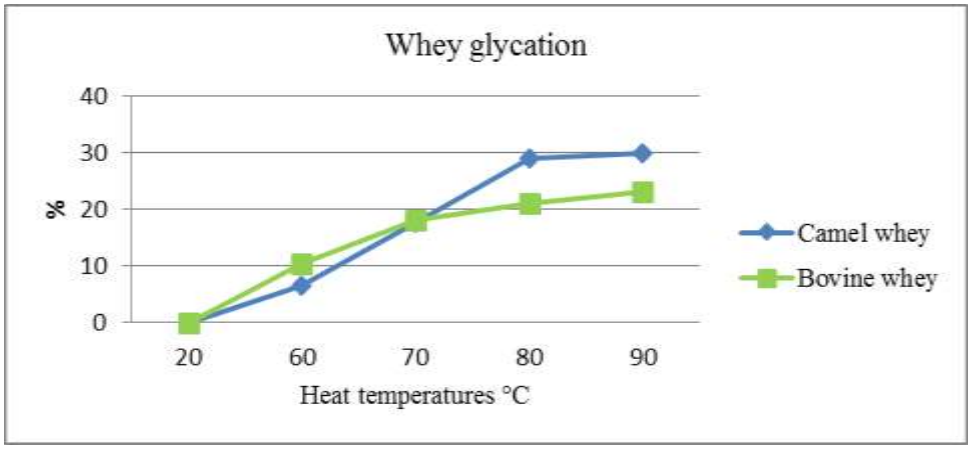

Figure 19 Increasing of glycation percentage as a measure of glycation degree for liquid whey, heat-treated at pH 7.

Increasing in glycation percentage reflects the reduced availability of free amino groups, as a result of the Maillard reaction. Figure 19 demonstrates that glycation was higher in camel whey as compared to bovine whey. This could be due to the higher protein

concentration in camel whey relative to lactose, leading to an increase in the Maillard reaction.

2.3.2.3.1 Glycation degree for skimmed milk fractions

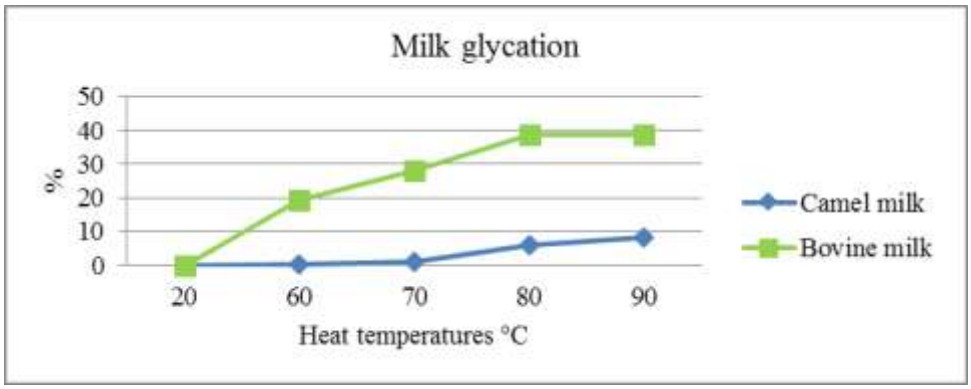

Figure 20 Increasing in glycation percentage as a measure of glycation degree for liquid skimmed milk heat.

Figure 20 indicates a higher glycation rate for bovine, as compared to camel, milk. This could be due to higher in lysine amino acid, which is less in camel milk than bovine milk, i.e. 6.6 and 8.1% of the total protein, respectively (Labuza, 2005). Lactose concentration is also higher in bovine milk as it was shown in Table 11.

2.3.3 Free and total SH groups of heat-treated milk fractions

2.3.3.1 Total SH profiles

2.3.3.1.1 Total SH for non -heated milk

The total SH in non-heated bovine milk was higher than that of unheated camel milk, possibly due to the lack of β lactoglobulin in camel milk. The µmolar SH/ g of protein , as calculated using the equation in section (3.3.5), amounted to 10.2 umol/g for bovine milk, i.e. similar to the value of 9.5 µml/ g reported by Owusu-Apenten (2005). The value for camel milk was 6.8 µmol/g litres, reflecting the lack of β lactoglobulin. The sulfhydryl groups could be attributed to that of camel serum albumin (CSA), one of the main proteins in camel whey that contains sulfhydryl groups (El Hatmi et al., 2014) Table 9. To the researcher's knowledge, this study had made the first report of the µmolar total sulfhydryl per gram of camel skimmed milk. Felfoul et al. (2015) measured the free sulfhydryl content of camel milk, but not the total SH groups.

2.3.3.1.2 Total SH for heated milk

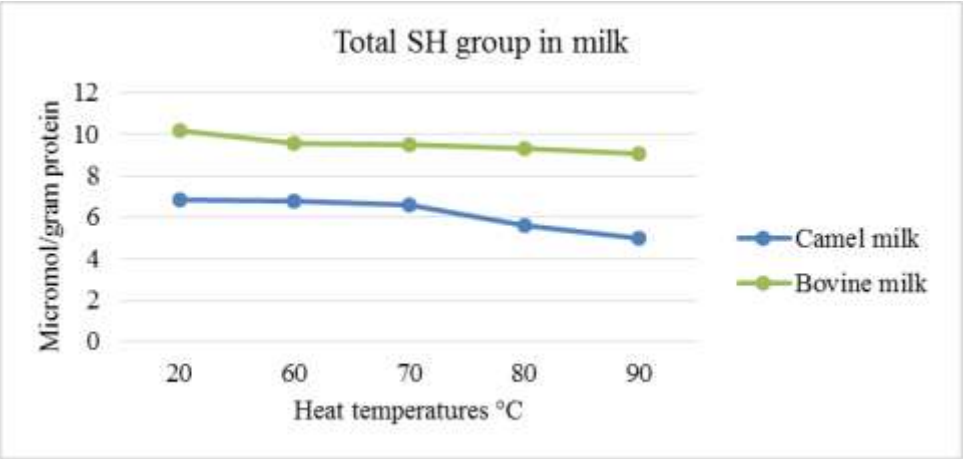

Figure 21 Total SH values for camel and bovine skimmed milk heated at neutral pH

Figure 21 demonstrates a stable SH profile for camel milk up to 70°C, declining significantly at 80°C, indicating denaturation. However, bovine milk reveals a comparatively smaller decline. This corresponds to results of Felfoul et al. (2015) who reported a Differential Scanning Calorimetry (DSC) endotherm of 77.8°C for camel milk and 81.7°C for bovine milk. The sharper decline in total SH for camel milk could also be due to the higher concentration of whey protein in camel milk (2.43%), as compared to bovine (i.e. 0.57%).

2.3.3.1.3 Total SH for camel and bovine and camel whey in pH 7

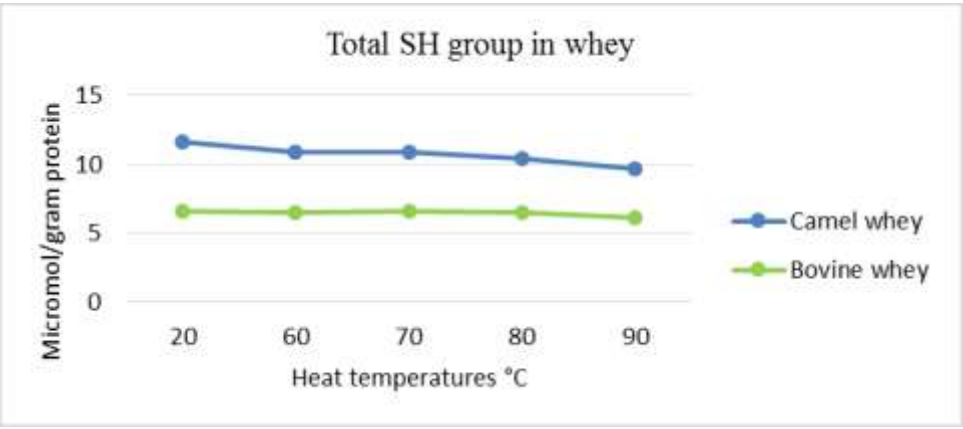

Figure 22 Total SH values for camel and bovine skimmed whey heated at neutral pH.

Total SH profiles for whey protein confirms the profiles for total SH of milk. Camel whey demonstrates a significant decrease in total SH at 80° C, similar to heated camel

skimmed milk. This contrasts with the findings of Laleye, et al., (2008), who reported a thermal transition peak as measured by DSC at 139°C for liquid camel whey. Although the current results indicate higher heat sensitivity for camel whey, in comparison to bovine whey, smaller increases in turbidity .indicate smaller increase aggregate size comparison to bovine whey. These findings indicate that camel whey is more heat sensitive to bovine whey, but is more heat stable.

2.3.3.2 Free SH profiles

2.3.3.2.1 Free SH for unheated milk

Figure 23 reflects a higher value of free SH groups for unheated camel milk whey compared to unheated bovine milk 1.94 and 1.35 µmol/g protein, resulting in a free SH/total SH ratio of 0.3 versus 0.13. The higher ratio could be due to the higher whey protein in camel milk in comparison to bovine milk Table 10.

2.3.3.2.2 Free SH for heat-treated milk

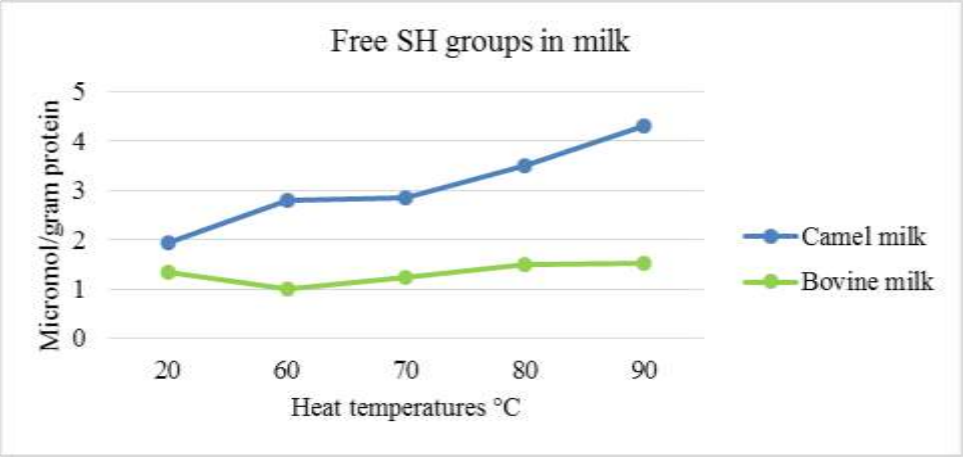

Figure 23 Free SH for camel and bovine milk heated at neutral pH

Camel milk demonstrates a significant increase in free SH above 70ºC, indicating an increased in denaturation. This supports Felfoul et al.'s (2015) findings in relation to denaturation temperatures of 77.8°C. The authors reported a higher value of free SH groups for bovine skimmed milk, as compared to camel skimmed milk. However, the difference in the current findings could be due to the fact that the tests were conducted with pasteurised camel and bovine milk. The higher ratio could be due to the higher whey protein in camel milk in comparison to bovine milk Table 10.

2.3.3.2.3 Free SH for non heated whey

Figure 24 demonstrates higher Free SH values for camel whey in comparison to bovine whey, resulting in a ratio of free to total SH of 0.12 versus 0.07 for bovine whey confirming the profiles for skimmed milk. This could also be due to the higher protein content in camel whey. Although β-lactoglobulin is absent in camel whey, the higher concentration of other globular proteins would account for the higher levels of free SH.

2.3.3.2.4 Free SH profile for heated whey

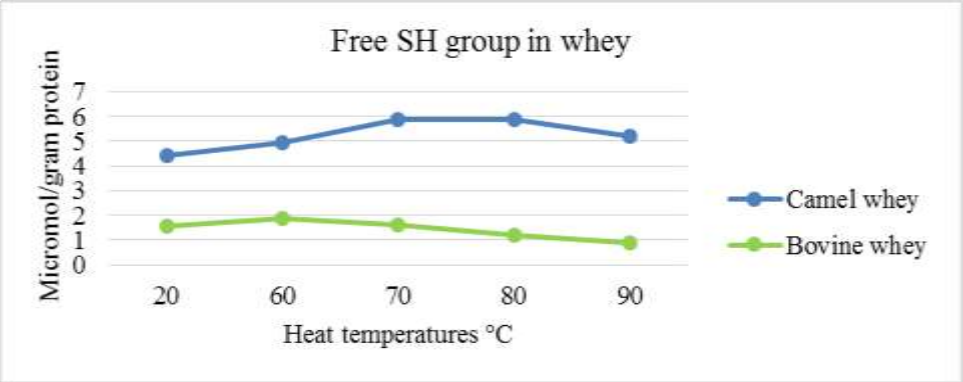

Figure 24 Free SH for camel and bovine whey heated at neutral pH

The free SH profile for bovine whey confirms the findings of Owusu-Apenten (2005), indicating a steady increase in free SH groups until 80ºC, followed by a decrease to 90ºC, due to the formation of disulphide bonds. Free SH for camel whey increases at 60ºC, demonstrated a sharp decline with an increase in temperature. This temperature sensitivity does not correlate with a lower increase in turbidity values than heated bovine whey and the higher solubility of heat-treated camel whey as compared to bovine whey. This could be due to the formation of smaller whey aggregates than in bovine whey, due to the absence of β-lactoglobulin in camel whey. This finding indicates that camel whey is more sensitive to heat treatment than bovine whey, while at the same time being more heat stable than bovine whey.

This investigation report here for the first time the heat sensitivity of camel whey in relation to free and total sulfhydryl group. The decrease in the free SH of camel whey at relatively low heating temperatures has the potential to influence on its nutritional properties. The knowledge reported here will prove to be of significant value for the commercial production of nutritional camel whey powders.

2.3.4 Analysis of non heated powders

Table 11 Chemical composition of bovine and camel skimmed milk powder as compared to published results of Suleiman et al. (2014)

Milk composition	Milk compositions		Published compositions (Suleiman et al., 2014)	
	Camel milk%	Bovine milk%	Camel milk%	Bovine milk%
Protein	37.36 ± 0.2	25± 0.7	26 -37	28 -33
Fat	9.31±0.42	2±0.21	n. a	1.5
Lactose	41 ± 0.1	47 ± 0.5	45-48	48-50
Ash	5.4 ±0.35	10±0.11	4- 7	7.-10

The protein content proved to be higher in camel than bovine skimmed milk, although both were found to be within the range of published values. However, the fat content for camel skimmed milk was higher than that of bovine skimmed milk (9.3% versus 2%). The removal of fat was found to be problematic in camel milk which could have been due to the small size of the fat globules (Farah and Rüegg, 1991).

The fat content for bovine skimmed milk has also found to be higher than the published values (i.e. 2% versus 1.5%), leading to the possibility that the fat was not fully removed, due to the sample not being sufficiently warm to separate all the fat content from casein. The lactose content for camel milk was slightly lower than the published value, inferring the possibility that different sources of milk could vary in composition.

Table 12 The chemical composition of bovine and camel whey as compared to published results (Suleiman et al., 20011; Canadian Dairy Commission, 2011)

Whey composition	Whey composition		Published whey compositions	
	Camel whey%	Bovine whey%	Camel whey%	Bovine whey%
Protein	55 ±0.07	12.07 ± 0.7	61.9%	10-13± 0.6
Lactose	41± 0.2	66 ± 0.53	unknown	70-75±0.34
Fat	3±0.43	1.5 ±61		0.5 ± 0.32
Ash	5.5 ±0.21	10.5.6 ±44		7-12 ±0.51

The camel whey protein was higher than bovine whey, at 55% compared to 12.07%. This confirms the values reported by Ali Al-Alawi and Laleye (2015). The camel casein composition is assumed to be similar to that of bovine casein composition which consists of protein concentration of about 85-91% and the rest of minerals (Southward, 2013).

2.3.5 Protein functionality in emulsion

2.3.5.1 Viscosity of emulsions made with powders

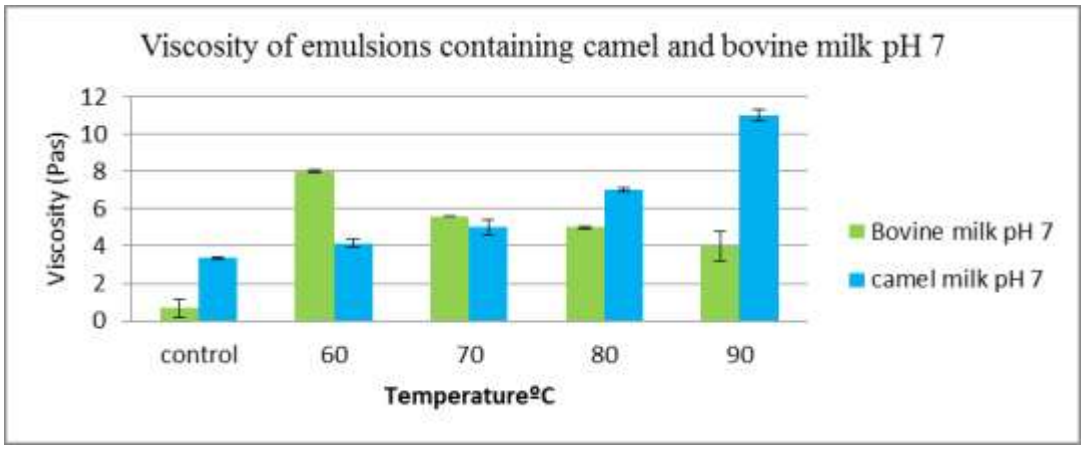

Figure 25 Viscosity of 60% oil emulsions made with pre heat-treated camel and bovine milk powder (37.5 mg/ml protein).

Figure 25 reveals that the highest viscosity results were produced by the bovine skimmed milk sample, when pre-heated to 60°C. The highest results for camel milk viscosity were found in samples pre-heated to 90ºC. The viscosity of emulsions is determined by emulsion oil droplet size and the water binding of proteins. Thus these results indicate that the viscosity of emulsions has the ability to be influenced by the particle size of the protein aggregates, i.e. a large increase in aggregate size (such as that for bovine milk heated to 80°C) could lead to decreased viscosity in comparison to the viscosity of aggregates formed at 60°C. Furthermore, camel milk reaches its maximum viscosity at 90°C, possibly as a result of the fact that smaller aggregates are formed in comparison to bovine milk heated to the same temperature (i.e. as demonstrated by the increase in respective turbidity .This investigation reports here for the first time the excellent emulsion viscosity enhancing properties of heat-treated (90 ºC) camel skimmed milk, in comparison to bovine skimmed milk heated at all temperatures apart form 60º C.

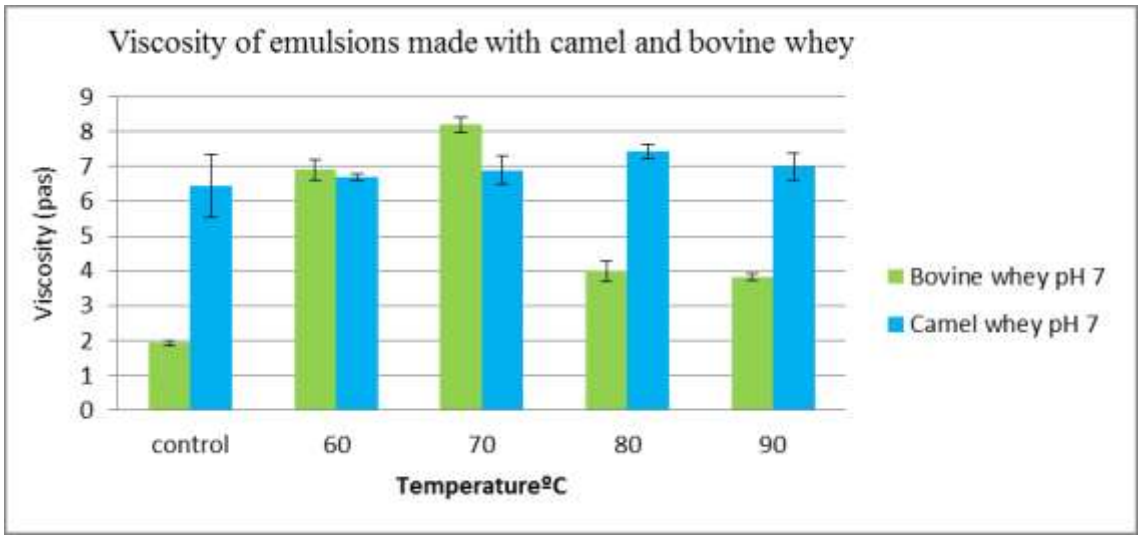

Figure 26 Viscosity of 60% oil emulsions made with pre heat-treated camel and bovine whey powder (37.5mg mg/ml) in shear rate 1

Figure 26 demonstrates that non-heated camel whey results in emulsion with significantly higher viscosity than unheated bovine whey at equal proteins concentration. Heat treatment of bovine whey results in increased viscosity of emulsions at 60ºC and 70°C, which declines at 80ºC and 90°C. This trend follows that of the increase and decrease in free SH group .Decrease in viscosity at a higher temperature (i.e. 90ºC) corresponds to a reduction in free SH, due to the formation of disulfide bonds and loss in functionality. The viscosity of emulsions in heat-treated camel whey remained constant up to 90ºC, thus confirming the heat stability of camel whey. This investigation reports here for the first time the excellent emulsification stability of heat-treated camel whey, as compared to bovine whey.

Laleye et al. (2008) prepared emulsions with liquid camel whey (2 mg/ml) which is a much lower concentration than the current study (37.5 mg/ml) and reported similar heat stability; however, there was no measurement of the viscosity or water separation of emulsions.

2.3.5.2 Oil droplet size distribution of emulsions made with milk and whey powders

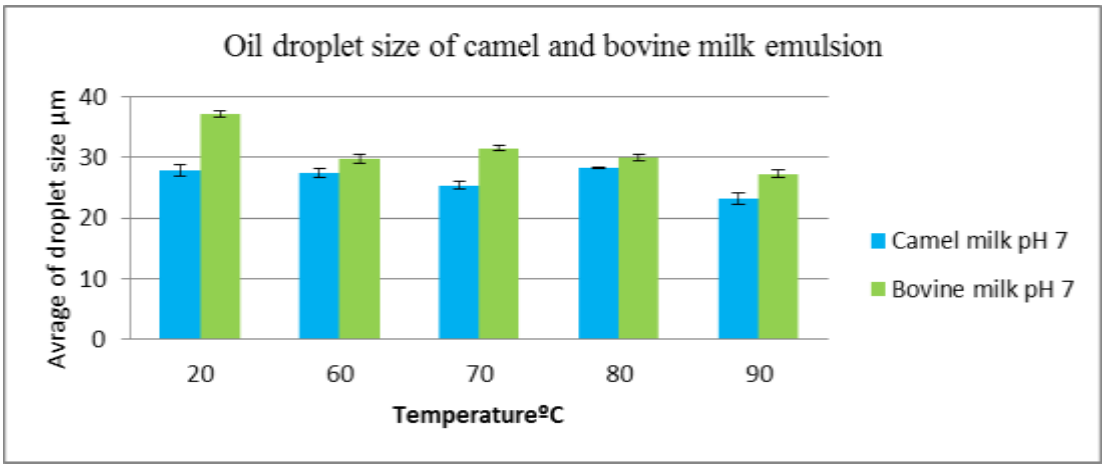

Figure 27 Average oil droplet size of emulsions (pH 7) made with skimmed milk that had been pre heated at different temperatures.

The measurement of the emulsion droplet size is an important indicator for the degree of emulsion stability. Good emulsion stability is characterised by small average droplet size and homogenous distribution**.** It will be demonstrated in figure 29 that stable emulsions are made with camel milk, suggesting a milk between mean emulsions droplet size and emulsion stability.

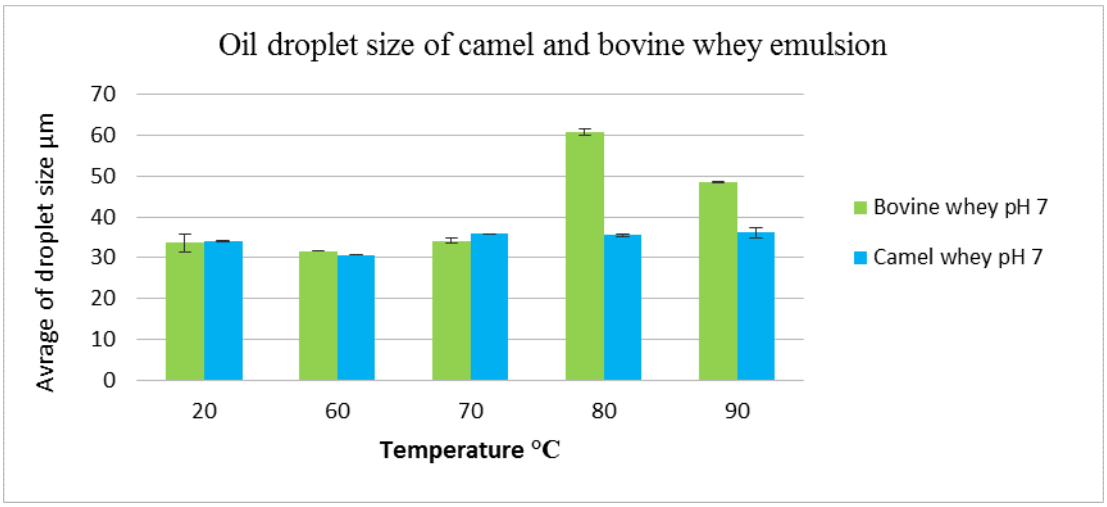

Figure 28 Average oil droplet size for emulsions (pH 7) made with whey powder that had been heated at different temperatures.

The results in Figure 28 reveal a significant increase in emulsion droplet size at 80ºC and 90°C with bovine samples. The droplet size of camel whey emulsions was found to

be stable at all heat treatment temperatures, thus confirming the results of Laleye et al. (2008). He found that the first marginal denaturation peak in bovine whey is due to β-LG, which is essentially absent in camel whey, while the second peak is due to the mixture of α-lactalbumin, serum albumin, and possibly part of the partially stabilized β-LG structure during the denaturation process. Because camel whey is deficient in β-LG, the denaturation peak at 139°C must be due to the mixture of α-lactalbumin and camel serum albumin. In both proteins, the highest thermal transition is due to sugars such as lactose.

2.3.5.3 Water-holding ability of emulsions

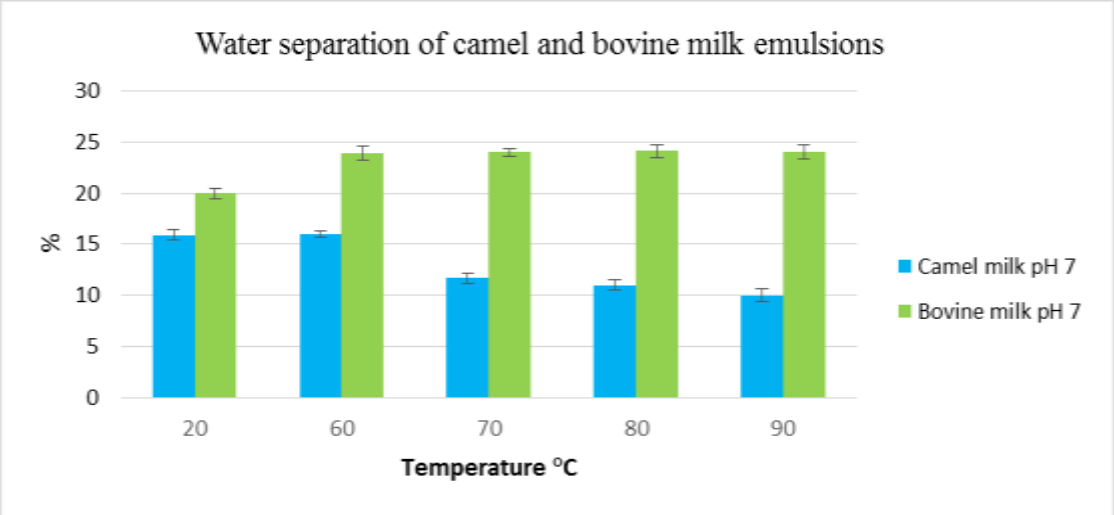

Figure 29 Water separations of emulsions made with camel and bovine skimmed milk pre-heated at different temperatures.

Emulsion made with bovine milk revealed increased water separation from temperatures of 60ºC and higher.wherease, camel milk samples were shown hgih improvement with increasing in heat treretment to ge the best the significant high water holding capacity in 90ºC. The hgih wate holding capacity of camel milk emulsion due to reduction in the interfacial tension between the hydrophilic and hydrophobic components in the milk. And camel whey was shown to have higher concentration of protein comapring to bovine milk Table 9, that could provide camel whey emulsion with significant low interfacial tension.

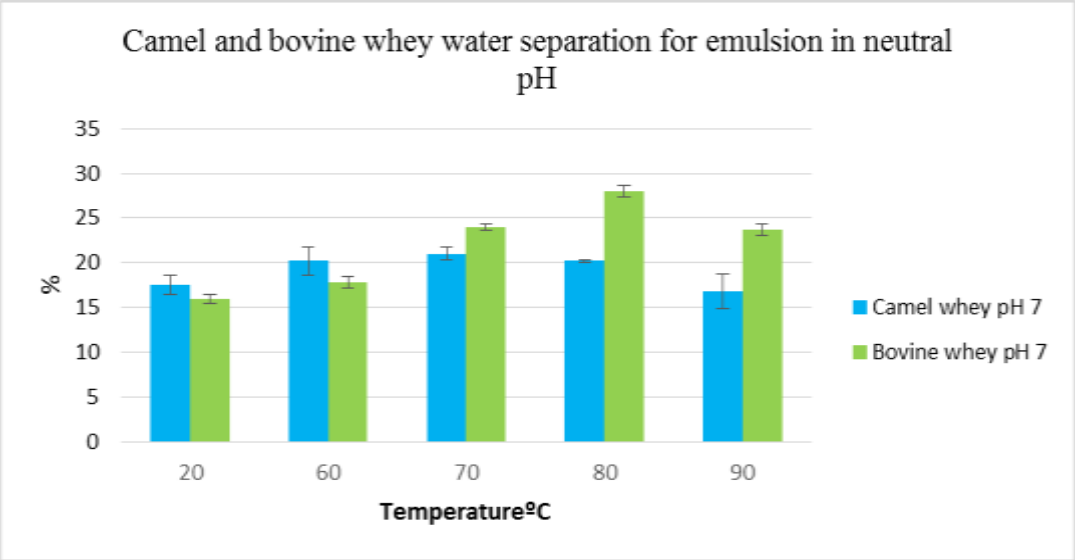

Figure 30 Water separation of emulsions for camel and bovine whey heat-treated at different temperatures.

The water holding ability (less water separation) remained relatively unchanged for camel whey, whereas bovine whey revealed increased water separation at 70ºC, 80ºC and 90ºC, which was higher than 20°C and that for camel whey at the same temperatures. As it has mentions before that, it could be camel whey was shown to have higher concentration of protein comparing to bovine milk Table 9, that could provide camel whey emulsion with significant low interfacial tension.

2.4 Conclusion

2.4.1 Novel findings

2.4.1.1 Freeze dried skimmed milk and whey

Proximate analysis of camel skimmed camel milk shows protein concentration were higher in camel milk than bovine milk (37.36 ± 0.2% and 25± 0.7% respectively): and fat was 9.31±0.42% and 2±0.21% respectively.

Proximate analysis of whey show protein concentration was higher in camel milk than bovine whey 55 ±0.07 and 12.07 ± 0.7 respectively: and fat it was 3±0.43 and 1.5 ±61 respectively. Camel casein was smaller in particle size compare to bovine casein.

2.4.1.2 Heat treated camel and bovine skimmed milk

2.4.1.2.1 Physicochemical properties

- Turbidity remained stable with both milk.
- Camel milk in liquid form solubility remained higher with heat treatment than bovine milk.
- Glycation was higher with bovine milk.
- Total SH groups in bovine milk was (4.8 umol/g) and for camel milk it was 3.2 μmolar SH/ g.
- The ratio of free to total SH was higher for camel milk (0.3) compared to bovine milk (0.14).

2.4.1.2.1 Functional properties

- Camel skimmed milk pre-heated to 90°C produced highest viscosity and lowest water separation.
- Camel milk emulsions droplet size were stable with all heat treatment and smaller than bovine whey and milk

2.4.2 Correlation between physicochemical and functional properties

The results indicate that camel skimmed milk is more heat stable than bovine milk as regards to functional properties. Ramet et al (2001) reported heat stability for camel milk and postulated that it could be due to the absence of β-lactoglobulin and low κ-casein. The higher ratio of free to total SH groups (0.3 versus 0.13), indicating that more free SH groups are available to form aggregates during heating. However there was no significant increase in aggregate size (stable turbidity and solubility), indicating

formation of small aggregates. This could be attributed to the lack of β-lactoglobulin preventing formation of large aggregates with itself or with κ-casein, as in heated bovine milk. The smaller camel milk aggregates could result in better emulsifying properties leading to higher emulsion viscosity than bovine skimmed milk. The higher concentration of denatured whey protein in camel milk could lead to higher water binding in emulsions at neutral pH compare to bovine skimmed milk, resulting in higher viscosity.

2.4.2.1 Heat treated camel and bovine whey

2.4.2.1.1 Physicochemical properties

- Turbidity increase was higher with bovine whey
- Glycation was higher with camel whey.
- Stable solubility compare to bovine whey
- Higher ratio of free to total SH for non- heated camel whey compared to bovine whey.

2.4.2.1.2 Functional properties

- Camel whey was heat stable and provided stable emulsions at all temperatures
- Camel whey emulsions droplet size were stable with all heat treatment and smaller than bovine whey

2.4.3 Correlation between physicochemical and functional properties

Low turbidity and stable solubility of camel whey results in stable emulsion with lower in droplet size and less water separation. The results confirm the findings of Laleye et al. (2008) for heat stability of camel whey in neutral oil/water emulsions. The higher free SH for camel whey and the sharp decline with heat treatment reveal heat sensitivity for camel whey, which is not expressed by in the formation of large insoluble aggregates which is the reason for its heat stability. However, the heat sensitivity would have an effect on nutritional value, as was found by reduction in insulin like activity of pasteurized camel milk (Chapter 5). We report for the first time that the total SH in non -heated camel skimmed milk protein is lower than bovine skimmed milk probably due to the lack of β lactoglobulin in camel milk

Camel milk was more heat sensitive than bovine skimmed milk, as indicated by higher increase in turbidity, decrease in solubility, and decrease in total SH and increase in free

SH compared to bovine skimmed milk. It was however more heat stable regarding functionality probably due to the formation of smaller aggregates than bovine milk. Liquid camel whey was more heat sensitive than bovine whey to heat treatment as regards to sulfhydryl groups, but is more heat stable as regards to solubility and functionality.

This investigation reports here for the first time the superior emulsion viscosity enhancing, water holding ability and oil droplet size reducing ability, of heat-treated (90 °C) camel skimmed milk, in comparison to heat treated bovine skimmed milk in an oil in water emulsion, pH 7. This investigation also reports here for the first time the excellent heat stability (at all temperatures) of camel whey as regards to emulsion (pH 7) water holding, oil droplet size and viscosity, as compared to heat treated bovine whey.

CHAPTER 3

DEVELOPMENT OF CAMEL MILK GELS, ACIDIFIED WITH GDL IN PREPARATION FOR DEVELOPMENT OF FERMENTED YOGHURT

3.1 Introduction

To produce set yoghurts, bovine milk is homogenised and heated at 85-95ºC for 10-30 minutes. The important of this step is to destroy the undesirable contaminant microorganisms. Also, it denaturizes inhibitory enzymes, that retard the subsequent yogurt fermentation. The heating time and temperature varies between manufacturers. The heated samples are then cooled to the fermentation temperature (42ºC), and inoculated with a starter culture. The type of yoghurt to be produced determines the processes that follow inoculation. For set yoghurt, the inoculated milk is loaded into consumer cups and incubated at the desired pH (4.5). The product is then cooled to 40ºC, without disturbing the curd (Guinee et al., 1995 and Guzman-Gonzalez et al., 1999). For stirred yoghurt, the inoculated bovine milk is poured into a tank and fermented. The gel is broken after fermentation, and the yoghurt cooled and pumped through a fine mesh, into cups.

The manufacturers of yoghurt from bovine milk usually fortify the yoghurt with dairy ingredients to increase the protein concentration to 40–50 g/kg. They may also add polysaccharides and stabilisers to improve the texture (Guinee et al., 1995; Guzman-Gonzalez et al., 1999). Traditionally, skimmed milk powders (SMP) are used to enrich the milk before fermentation. However, the improved quality and availability of alternative dairy ingredients, such as whey protein concentrates (WPC), produced by ultrafiltration, provides a cost-effective alternative to SMP. In addition, whey proteins deliver different functional properties to the final product, when compared to whole milk proteins from SMP (Guinee et al., 1995; Guzman-Gonzalez et al., 1999).

Several studies have investigated the impact on the textural and physical properties of bovine yoghurt products when replacing SMP with WPC. Some report that the WPC increases the yoghurt's firmness and/or viscosity. In contrast, studies by Guinee et al. (1995) and Guzman-Gonzalez et al. (1999) report that WPC contributes to a similar or weaker viscosity than SMP. The effects of WPC on the water-holding capacity of yoghurt are also inconsistent. Guzman-Gonzalez et al. (1999) and Chandan et al. (2008) show that yoghurts containing WPC have a greater water retention capacity than those enriched with SMP; although Guinee et al. (1995), Modler et al. (1983), and Remeuf et al. (1989), report the opposite. These conflicting results may be because these studies used different methods to determine the physical and rheological properties of the bovine yoghurt. Some methods focus on viscoelastic behaviour (in which the bovine

yoghurt structure is not affected), whereas others evaluate the yoghurt's flow behaviour (in which the initial bovine yoghurt structure is altered). Secondly, the use of different starter cultures to ferment the bovine milk source might also influence the yoghurt's properties. Finally, functional properties vary among different commercial WPCs, which may also contribute to some of the inconsistencies observed between studies. In recent years, advanced processing techniques have improved the consistency and functionality of WPC, although variations in the properties of WPC-fortified yoghurts still exist (Sodini, Montella, and Tong 2007).

Camel milk yoghurt has not been produced commercially yet, primarily because camel milk is only available in certain areas, such as Africa and the gulf areas of Asian countries. Due to the low casein content in camel milk (1.45%) compared to bovine milk (2.85%), attempts to obtain a firm gel with acidified camel milk without additional additives, have been unsuccessful. Making yoghurt from camel milk has been attempted by previous researchers (Otaibi, 2013; Shori and Baba, 2011; Hashim at el., 2009; Edrees, 2013; Jumah, et al., 2001). However, they have all required the use of additives such as bovine SMP and/or several commercial stabilisers. Because low acid coagulation properties of the casein in camel milk versus bovine milk, the chemical composition of camel milk, namely total solids and protein content, influences the rheological properties of the curds formed during yoghurt fermentation, i.e. during gradual acidification by lactic acid bacteria (Jumah et al., 2001). The aim of the work reported in this chapter is to develop an acidified gel comprised mostly of camel milk ingredients.

3.1.1 Casein precipitation

Throughout the stage of acidification of bovine milk, progressive neutralisation of the electric charges of the casein micelles occurs, leading to the emergence of curd. In camel milk, it is difficult to detect a similar development, because the formation of the curd is slow, unstructured and resembles a floc rather than a precipitate (Farah and Bachmann, 1987).

The optimum pH for bovine casein precipitation is pH 4.6, and it is 4.3 for camel casein (Wangoh, Farah; Puhan, (1998); Kappeler et al. (1998). A well-known model proposes that bovine casein micelles are composed of sub micelles, linked together by calcium phosphate bridges and hydrophobic interactions (Zhong et al., 2007). The "hairy" κ-

casein layer on the micelle surface provides strong repulsive steric interactions, which prevent casein aggregation. Lowering the pH to the isoelectric point of casein (pH 4.6) diminishes the net electrostatic charge and repulsive steric interactions, enabling the aggregation of casein micelles in the production of yogurt (Aimutis, 2004). A small change in the pH significantly alters the quality and functionality of casein gelation products (Zhong et al., 2007; Janer et al., 2004).

Ramet (1990), investigated the additional of calcium and sodium to camel milk to improve casein precipitation. However, no improvement was found compared to lactic and citric acids. The only way to separate casein after acidification is to centrifuge it, which allows a watery concentrate to be recovered with about 16 to 22% total solids. This could be because camel casein is lower in κ-casein than bovine casein; they are 0.07% and 0.3% respectively in liquid milk (Ramet, 2001).

Most formulations for the preparation of yoghurt use hydrocolloids to enhance thickening. Hydrocolloids are colloidal substances with an affinity for water, in which they produce viscous solutions, pseudo-gels, or gels; they are macromolecular hydrophilic substances. Some of them are water-soluble and form colloidal solutions; others are only able to swell in water and can be dispersed by means of shear forces. The heterogeneous group consists of polysaccharides and proteins (Wüstenberg, 2014).
Hydrocolloids are used in technical and regulated applications to thicken and stabilise formulations. In processed foods, they are ubiquitous, that is no other group of ingredients contributes more to viscosity, texture, and body (Wüstenberg, 2014).

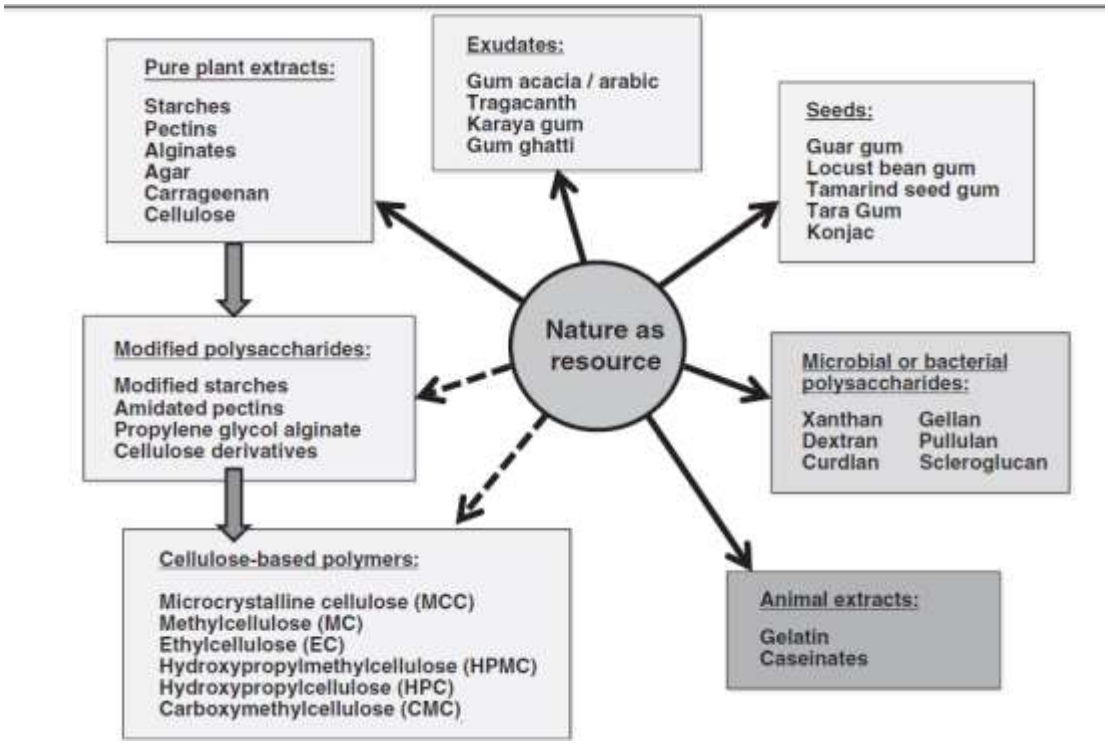

Figure 31 Overview of food hydrocolloids used globally. (Wüstenberg, 2014)

A recent study by Khalifa and Ibrahim (2015), involved the preparation of camel milk yoghurt, with the additions of bovine gelatin E441, mono and diglycerides of fatty acid E471; guar gum E412, sodium carboxymethyl cellulose E466 modified starch E1422, mono and diglyceride fatty acid E471, to fresh camel milk. This made it possible to provide high viscosity and prevent separation of the serum (Khalifa and Ibrahim, 2015). In this chapter, we chose to use agarose (seaweed gelatin) as a stabiliser for the yogurt. Agar is a neutral hydrocolloid (extracted from seaweed). It is a jelly-like substance, obtained from algae, and was first discovered in Japan, where it is called *kanten*. Agar is derived from polysaccharide agarose, which forms the supporting structure in the cell walls of certain species of algae, which are released on boiling. The greatest advantage of agar in different food applications derives from its characteristic firm texture, heat tolerance, stability under acidic conditions, high solubility in concentrated sugar solutions, and limited reactivity with other food components.In addition agarose is the most acceptable stabilizer for Saudi community, they have been apply it with sweets.

3.1.2 GDL (Glucono-delta-Lactone)

GDL is a white crystalline powder, and a neutral cyclic ester of gluconic acid. This form of acid is produced by the aerobic fermentation of a carbohydrate source. After fermentation, the carbohydrate is purified and crystallised into GDL. GDL is usually extracted from plant sources, and is commonly used to investigate changes that occur in

casein micelles during acidic gel formation (Vasbinder et al., 2003). GDL is an organic acid in solid form, similar to citric acid. The acidogen turns to acid, gradually through hydrolysis in an aqueous medium. When added to an aqueous solution, GDL dissolves rapidly, and then hydrolyses progressively to gluconic acid, causing the taste to change from slightly sweet to mildly acidic. It is GDL's gentle acidification and mild taste that separates it from other acidulates; thus, it is preferred in applications requiring a controlled reduction in pH. It ensures the milk can be acidified slowly and homogenously to a desirable pH, i.e. below the isoelectric point, and then the casein is gelled.

3.1.3 Principles of analytical methods used in this chapter

3.1.3.1 SDS PAGE (Polyacrylamide Gel Electrophoresis)

"SDS PAGE is an analytical method used to separate components of a protein mixture according to their size. The technique is based on the principle that a charged molecule will migrate in an electric field towards an electrode with the opposite charge. Electrophoresis techniques cannot generally be used to determine the molecular weight of biological molecules, because the mobility of a substance in a gel depends on both charge and size. To overcome this, biological samples need to be treated so that they acquire a uniform charge, then the electrophoretic mobility depends primarily on size. To establish a difference, protein molecules need to be denatured with SDS. This means that the proteins then lose their secondary, tertiary or quaternary structure. The proteins are negatively charged by the effect of the SDS, and when loaded onto a gel and placed in an electric field, will migrate towards the anode (positively charged electrode), where they can be separated by a molecular sieving effect based on size. After visualisation using a staining (protein-specific) technique, the size of a protein can be detected with that of a known molecular weight marker" (copied fromWalker, 2002).

3.1.3.2 Confocal microscopy

The object and its image are "confocal." The microscope is able to filter out the out-of-focus light from above and below the point of focus. Light from above and below the plane of focus is thus eliminated from the final image. Another useful feature of confocal microscope is the ability to show co-localisations of signals from different fluorochromes. In specimens double-labelled, for different structures, the different fluorochromes can be collected in different channels and combined to make colour images, which along with the three dimensional information obtained by confocal

sectioning can more precisely show co-localisations of signals than with a normal fluorescence microscope (Ryther, 2015).

3.1.4 Aims of this chapter

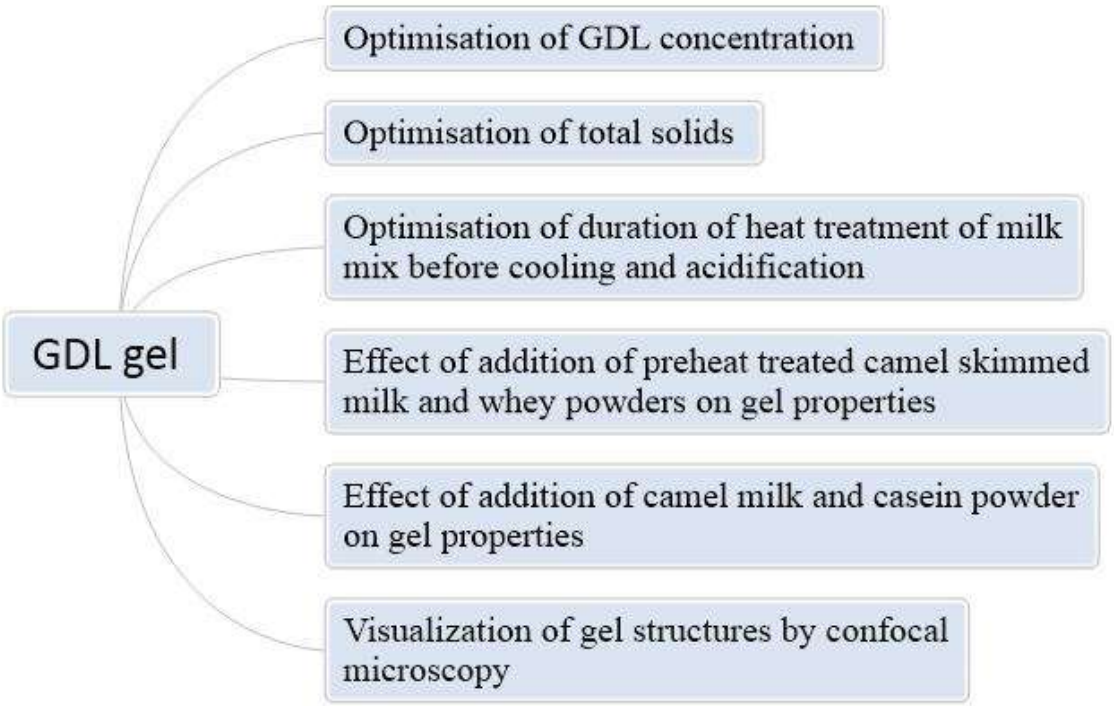

Figure 32 Aims of this chapter

The main objective of this chapter was to develop an acid gel to serve as basis for the development of fermented yoghurt. To simplify the optimisation of gels for yoghurt development, we chose to test the gels acidification processes. In this study, GDL was used instead of lactic acid bacteria.

3.2 Material and methods

3.2.1 Materials

- GDL (D-(+)-Gluconic acid δ-lactone was obtained from Sigma Aldrich, cat no: G4750.
- Agarose (seaweed gelatine): Ceylon moss (Gelidium amansii) – common name china grass or agar, was obtained from Intralabs, UK acetic acid.
- Raw camel milk was obtained from Bradford, UK (Kamelus, the home of "White Gold").
- This source was discontinued, and the decision was made to source pasteurised camel milk from Saudi Arabia (al-turath al- Saudi), which was exported to Edinburgh frozen, in order to continue with experiments.
- Bovine pasteurised skimmed milk was bought from the local supermarket (ASDA Fresh Milk Skimmed).
- camel skimmed milk powder (non-heated and preheated) camel whey powder (non-heated and preheated)
- Camel casein powder none heat-treated.
- pH meter.
- Acetic acid, sigma, cat no: A6283.
- Rhodamine B sigma, cat no: R6626.
- 2x Laemmli Sample Buffer Bio-rad, cat no: 161-0737EDU.
- Tricine Sample Buffer Bio-rad cat no: 161-0739.
- Bolt® 4-12% Bis-Tris plus Gel, 10 well, cat no: BG04120Box.

3.2.2 Methods

3.2.2.1 Optimising conditions for acid gel formation

3.2.2.1.1 Acid gelation with acetic acid

The effect of the addition of acetic acid: liquid camel and bovine milk were acidified to different ranges from pH 6.6 to 3 of pH with 20% acetic acid. The samples were then heated at 90°C for 30 minutes. Gel formation was observed visually.

3.2.2.1.2 Acid gelation with GDL

Different concentrations of GDL were added to camel and bovine milk; the pH gel was measured using the pH meter at the different times to obtain the best gel curd.

3.2.2.2 Effect of total solids

To increase the total solids in the camel milk; different concentrations of agarose Table 15 chapter 3, camel whey powder, and camel skimmed milk, were added to the camel milk, and stirred for 30 minutes using a magnetic stirrer.

Casein powder was added to the liquid camel milk, and the pH adjusted to 7 with 1 M Na OH and stirred for 30 minutes using a magnetic stirrer.

3.2.2.3 Effect sequence of GDL addition and heat treatment

Effect of the addition of GDL after heat treatment:

Milk mix B (see Table 13 chapter 3 was heated in 90ºC for 30 minutes. The sample was cooled to 25°C, 4% GDL was added, followed by incubation at 40°C until the pH reached 4.3.

Effect of the addition of GDL during heat treatment:

4% GDL was added to Milk Mix B (Table 13) and heat treated as above.

Effect of the addition of GDL without heat treatment:

4% GDL was added to Milk Mix B (Table 13) followed by incubation 40°C until the pH reached 4.3

3.2.2.4 Effect of the addition of heat treated camel whey and skimmed milk powder on the texture of the GDL gel.

Camel whey (that had been heat treated at 80 ºC) and skimmed milk fractions that had been heat treated at 90ºC, followed by freeze-drying as prepared in Chapter 2 section 3.2.1, were tested, based on the recipe Mix Table 13

Process A was followed (see Figure 42)

The samples were heated at 90ºC for 45 minutes, cooled on ice to 40°C, then 4% GDL was added, followed by incubation at 40°C until the pH reached 4.3. Gels were stored at 4°C for at least 12 hours before further tests were carried out.

3.2.2.5 Effect of addition of camel casein

Casein powder was added to the liquid camel milk at different concentrations, and the pH was adjusted to 7 with 1 M NaOH. The solution was blended for 30 seconds with a hand blender, and stirred for 30 minutes using a magnetic stirrer.

3.2.2.6 Effect of duration of heat treatment

Heat treatment was applied to Mix B (Table 13) in 15, 30, 45, and 60 minutes in 90ºC. Followed by the acidification process has described in Figure 42

3.2.2.6.1 Texture measurements

Triplicate samples of yoghurts were compressed to 50% deformation, using a Zwick/Roell type Z010 machine. A speed test cycle is 10mm/min.

3.2.2.6.2 Rheological measurements (viscosity)

The viscosity of the samples was measured with a rheometer, as previously described in section 2.2.6. The storage modulus (G') was measured at a constant strain in the linear region, at 4°C for 10 minutes. To measure the development of viscosity during GDL acidification, the 4% GDL gel was added to the liquid bovine and camel milk in the measurement chamber. The viscosity was measured according to time at viscoelasticity of 1 Hz. During the acidification process the gel was measured at a fixed temperature (40ºC) for 80 minutes, to mimic the yogurt making temperature.

3.2.2.6.3 Confocal laser scanning microscopy

Samples were stained with Rhodamine B (0.2 wt. % solution; 10µl per ml sample) to visualise the protein phase (Sala, 2007; Fagan et al., 2006; Vasbinder et al., 2003). Lenses' magnifying type 10X.

3.2.2.7 Measurement of water holding ability of gels

Samples were (centrifuged at 10,000 rpm for 20 min, the water released from the emulsion was then measured as follows

Emulsion quantity was (ml)/gel quantity before incubation*100.

3.2.2.8 SDS PAGE

SDS-PAGE was carried out according to the procedure of Wu and Hojilla-Eva (2005) mehtod, by using Pre-cast native PAGE 12% Tris-glycine gels in an electrophoresis unit (XCell Surelock™ Mini Cell, Invitrogen Life Technologies, Paisley, UK), at constant voltage 200V for approximately 45 to 60 min. Samples were prepared in ready to used non-reducing sample buffer (120M Tris-HCl, pH6.8, 20% glycerol, 4% SDS, and 0.008% bromophenol blue; while reduced sample buffer added 10% β-Mercaptoethanol). Running buffer was 10x SDS-PAGE buffer (1% SDS, 0.25M Tris-HCl and 1.92M glycine per liter). Samples were run under reducing conditions (2-mercaptoethanol), and non-reducing conditions.

3.3 Results and discussions

3.3.1 Gelation properties of camel milk following acidification with acetic acid

The acidity of milk gel should be in the range was 4.2 to 4.6 pH, as Hakimi et al (2014) have observed. Otherwise, the sensory acceptability of the milk gel would be undesirable to consumers.

In the following experiment, a range of pHs was applied to camel and bovine milk (liquid form) to investigate the effect of pH for gel forming in both samples. Camel and bovine milk were acidified, from pH 6.6 to 3. The acidification step was applied using 20% acetic acid, and then heated at 90ºC for 30 minutes. The purpose of this step was to investigate the optimal pH for achieving a curd in camel milk and bovine milk.

Figure 33 Visual appearance of bovine milk acidified with acetic acid at different pH values.

Figure 34 Visual appearance of camel milk acidified with acetic acid to achieve different pH values.

The results illustrate that bovine milk at pH 4.8 and 5 form better gel curt than those of the camel milk prepared under the same conditions. Furthermore, the camel whey, following acidification of the milk, was significantly turbid compared to acidified bovine milk, which could be attributable to the fat suspension in the camel whey (Hakimi, Rohani, and Hemmatboland, 2014).

3.3.2 Camel and bovine Milk gelation properties acidified with GDL

3.3.2.1 Effect of GDL concentration on pH of gels

Each measurement was done in triplicate. Standard deviation was calculated using Excel. The errors bar values were too small to display on the chart <0.01.

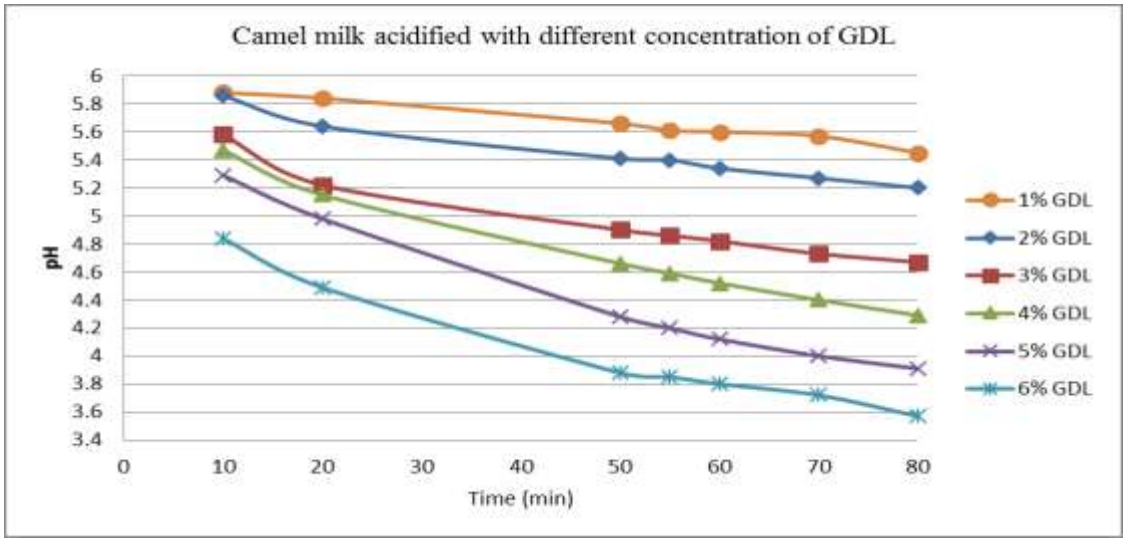

Figure 35 Acidification profile of full fat camel milk with different concentrations of GDL

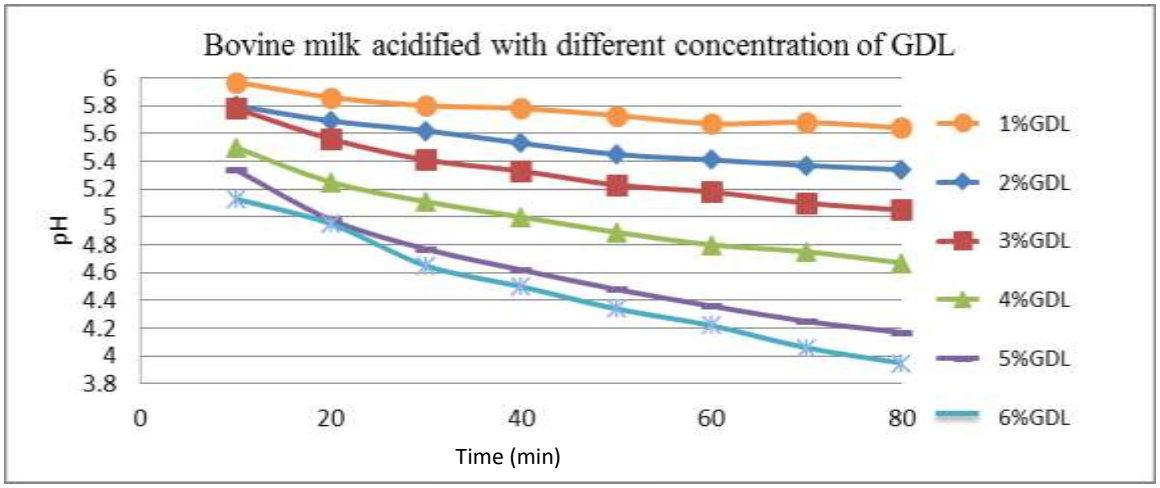

Figure 36 Acidification profile of full fat bovine milk, with different concentrations of GDL

Figure 36 and 35 reveal that the pH value of the milk decreased after adding higher concentrations of GDL. The results also indicate that 1 and 2% of GDL added to camel milk resulted in the same level of pH decrease in both camel and bovine milk. However, camel milk with 3% and 4% of GDL demonstrates a dramatically faster decrease in pH

compared with bovine milk. In relation to the addition of a 5% and 6% GDL concentration, the bovine milk did not show a significant effect on the rate of pH decrease; whereas, camel milk under the same conditions showed a dramatic decrease in pH values. The high decrease in camel milk pH comparing to bovine milk could be demonstrated by the lack of κ.casein, which is the responsible of the casein stability in milk.

3.3.2.1 Rheological measurement

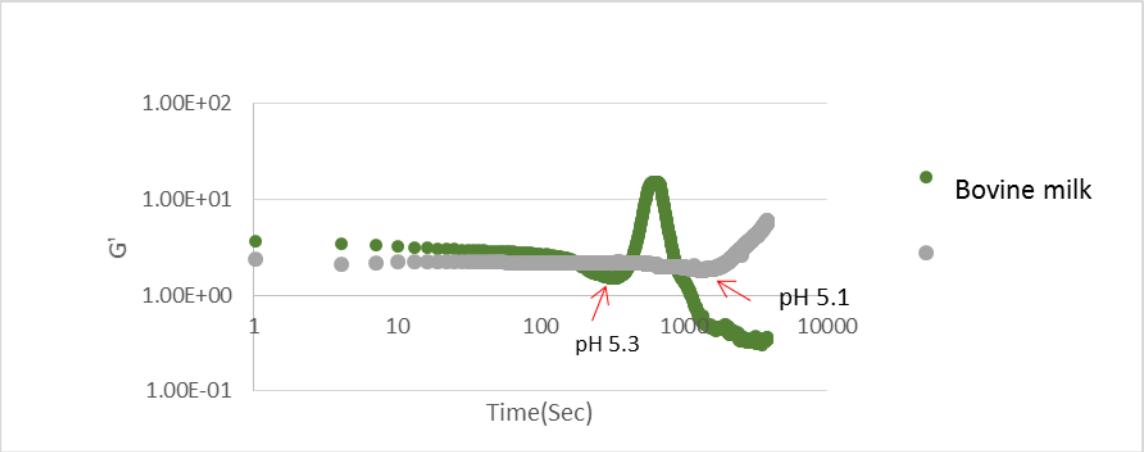

Figure 37 Development of gel hardness (G'), with time for both camel and bovine milk with a GDL concentration of 4%

The bovine milk profile in Figure 37 shows a sharp peak in increase in gel hardness after 9.3 minutes acidification in pH 5.3. This increase would correspond with the iso electric pH for bovine casein pH 5.3. The results in Figure 37 show a somewhat longer time for 4% GDL to reach 4.6 which could be due to experimental variability. The curve shows weakening of gel at lower pH (longer incubation time). It is known that a bovine cheese curd becomes weaker at pH below iso electric pH (Fox et al., 2004). On the other hand, the viscosity of camel milk gels starts to increase after 17.7 minutes in pH 5.1 and is still increasing after 80 minutes of being incubated. Figure 37 shows that camel milk sample needs more incubation time to reach the same bovine milk gel curt, that could be demonstrated by the absent of β Lactoglubolin, which consider as the main component that contained the highest concentration of SH groups to form disulphide bonds and that lead to forming gel. The major protein in camel milk is whey, which is different than in bovine milk. And that could affect the process of forming the curt in camel milk gel.

These results confirm the results shown in Figure 37 which reveal that the iso electric pH of 4.3 for camel milk acidified with 4% GDL is reached after 80 minutes.

3.3.2.2 Confocal Laser Scanning Microscopy of gels

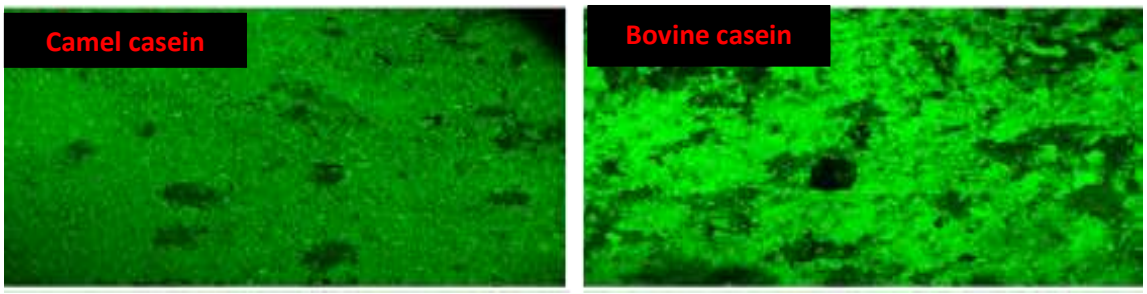

Figure 38 Confocal microscopy photographs of GDL acidified camel milk (pH 4.3) and bovine milk (pH 4.3)

The variation in gel structure, between camel and bovine milk samples acidified with GDL, is clearly visible. The results revealed that the gel structures that formed in bovine milk were large and coagulated, whereas, the form of the camel milk gel structure was much finer coagulates. This result could be attributed to the κ-casein concentration in bovine milk, which is higher than that in the camel casein sample, as outlined above (see Table 9 Chapter 2). In addition, in the case of bovine milk, β-Lactoglubolin interacts with the κ-casein on the surface of the casein micelles during heat treatment (Lee and Lucey, 2004), but there is no β- Lactoglubolin in camel milk. This fact could explain the different gel formations in the two types of milk (Schorsch et al., 2000; Jaros, 2013; Law and Tamime, 2010; Vasbinder, Rollema et al., 2003; Trejo, 2012).

3.3.3 Increasing total solids of camel milk

Camel milk was prepared with various concentrations of total solids, with the addition of unheated skimmed milk powder, unheated whey powder, unheated casein and sea weed agararose. The samples were stirred for 30 minutes, and followed by the addition of 4% GDL, and then incubated at 40ºC to pH 4.3. Increasing total solid gives and increasing in viscosity. To get the optimal viscosity was obtained with two-fermented yogurt samples show in Table 13.

Table 13 Optimal recipe for making camel milk yogurt.

Ingredient	Total solids%	
	Mix A	Mix B
Camel full fat milk	6.5	6.5
Seaweed agarose	1	2
Camel skimmed milk powder	0	1
Camel casein	4	0
Camel whey powder	0	1
GDL	4	4
	15.5	14.5

We found that the addition of 2% seaweed agarose was essential to obtain a solid gel using skimmed milk powder and whey powder with a GDL acidified sample (Mix B). It was possible to decrease the concentration of agarose to 1% with casein (Mix A), however, at 1% increased total solids, which would lead to higher costs to the consumer. We decided to use the Mix B recipe as a basis for further development work in this chapter. The protein composition in the recipe was demonstrated in Tables 9, 11 and 12, and that was 0.185, 0.372, 3.52 and 0.55% of protein provided by camel full fat milk, camel milk powder, camel casein powder and camel whey powder respectively. So that total protein concentration was used in the formula was 4.627%.

3.3.4 Effects of the sequence of the addition of GDL and heat treatment on the gelation visual appearnce

Mix B (see Table 13), was heat treated at 90 ºC for 30 minutes and then cooled on ice to room temperature. The only difference between the samples was the sequence of the addition of GDL.

Figure 39 Visual appearances of gels formed by the addition of GDL before or after heat treatment.

The results shown in Figure 39 indicate that the best gel texture was obtained when GDL was added after the heat-treated milk mix has cooled (sample 1). This sample formed a firmer gel than sample 2, and showed no water separation. Sample 3, which was a combination of all ingredients without any heat treatment, did not form a gel, and shows casein precipitated from whey. These results emphasise the importance of heat treatment in order to form an acid gel.

3.3.5 Effect of the duration of heat treatment of milk mixture at 90ºC before acidifcation with GDL

Table 14 Texture, viscosity and synteresis of Milk Mix B heat treated for different periods at 90ºC before cooling and acidification.

Samples	Heating periods min	Syneresis %	Hardness Newton	Viscosity /1 shear rate
5	15	60	0.07±0.1	0.5 ±0.3
6	30	50	0.09±0.5	1.01±0.3
7	45	15	0.28±0.3	16.96±0.2
8	60	10	0. 337±0.7	22.4±0.3
Commercial yogurt			0.90 ±0.61	11.4±0.5

The texture, water separation, and appearance of sample 7 (Table 14), which was heat treated for 45 mines, had the most acceptable gel properties based on gel clot that obtained, with a gel hardness of 0.28±0.3. The gel hardness of commercial yogurt textures generally ranges from 0.25–0.90 N (Rosenthal, 2010). Viscosity was also in the region of that of the commercial sample. Sample 8 shows a significantly higher viscosity than the commercial yogurt sample and sample 7.

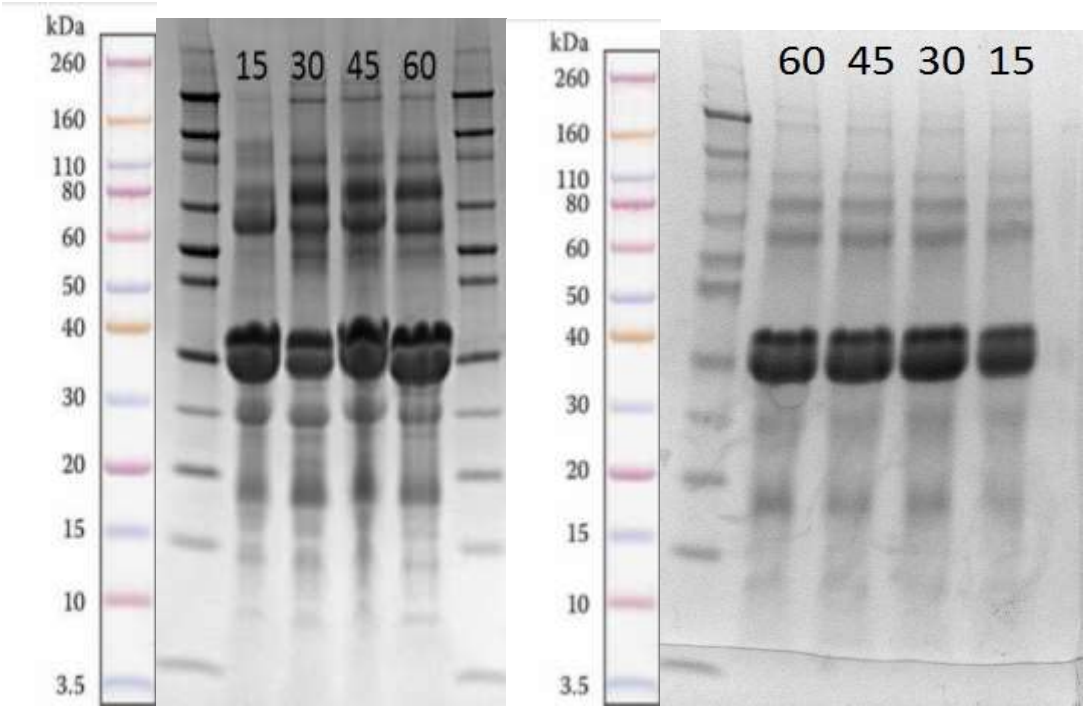

Figure 40 SDS-PAGE (10–20% gradient) stained with Commassie Blue of milk Mix B heat treated for different lengths of time before acidification,. The figure on the left shows the profile of the non-reduced samples and the figure on the right shows the profiles of the reduced samples

The non-reduced gel indicates that higher molecular weight bands had formed in response to 30, 45, and 60-minute heat treatments, compared to the 15-minute heat treatment of mixed yogurt, which showed no similar bands. The reduced gel (right) reveals that most of the higher molecular weight bands resolved to similar bands as those in the 15 minute heated sample; although bands of higher intensity >80 KD appear at 45 and 60 minutes, indicating that compounds with a higher molecular weight are formed by disulphide bonds formation. Including 2-Mercaptoethanol in the reducing gel, breaks the disulphide bonds.

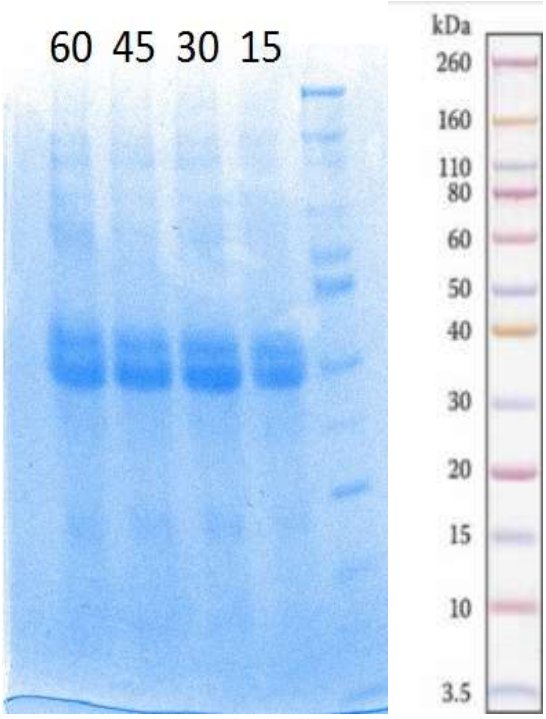

Figure 41 SDS-PAGE (10-20% gradient) stained with Poncea (glycoprotein stain) of Mix B (reduced) heat treated for different l. The figure on the right is a profile of pre stained molecular weight markers left shows the profiles of non-reduced samples

The reduced gel demonstrates that a sample heated for 54 and 60 minutes shows bands in the region of 80 KDa, indicating the formation of glycated polymers by covalent bonds that cannot be broken with a reducing agent. These results show that the increased heat treatment of the milk mixture before acidification leads to increased disulfide bond formation and glycation reactions, forming large aggregates. Upon acidification the aggregates will co-precipitate with casein and other denatured whey proteins, entrapping more water and fat and forming a denser network.

3.3.6 Final process for preparation of GDL gels

We decided to apply the following procedure to prepare the GDL gels.

Process A

1) Stirring of ingredients for 30 minutes using a magnetic stirrer.
2) Heating of milk mixture in closed Pyrex bottles to provide milk from being evaporated for 45 minutes at 90°C (Figure 14) to deactivate the anti-microbial activity in camel milk. And it was covered to avoid water evaporated by heat
3) Cooling on ice to 40°C before fermentation process to make sure it's not too hot for microbial culture, otherwise they would be destroyed by heat
4) Addition of 4% GDL, the concentration of GDL was applied is based on Figure 37 and 38
5) Incubation at 40°C till sample reach the pH 4.3 (the optimal pH for curt formation)
6) Storing at 4°C for at least 12 hours
7) Measurement of physical properties.

Figure 42 Process for preparation of GDL gels (Process **A)**

3.3.7 Effect of addition of preheated skimmed milk and whey powder on gel properties

The Mix B Table 13 was reformulated with preheated skimmed and whey protein, with the aim of reducing the concentration of seaweed agarose. Process A was followed to prepare the gels and measure their physical properties

The most meaningful results are reported in Table 15.

Table 15 Effect of skimmed milk powder, whey powder and agarose on gel properties

Sample	Agarose %	Whey powder %	Milk powder%	Syneresis%	Texture Newton	Total solids %	Viscosity Pas
1	0	4	0	Very soft	Very soft	14.5	Very low
2	0	0	4	Very soft	Very soft	14.5	Very low
3	0	2	3	Very soft	Very soft	15 .5	Very low
4	1	4^{x}	0	30±0.3	0.2±0.8	15.5	12.47±0.03
5	1	4^{v}	0	30±0.7	0.29±0.2	15.5	14.04±0.01
6	1	0	4^{x}	25±0.4	0.31±0.3	15.5	17.24±0.02
7	1	0	4^{z}	15±0.2	0.94±0.6	15.5	27.59±0.1
8	0.5	3^{y}	2^{z}	15±0.8	0.54±0.4	16	23.44±0.04
9	0.5	4^{y}	1^{x}	27.5±0.6	0.16±0.1	16	5.69±0.6
C				0	0.96		11.4±0.3

C= commercial yogurt; X non heated; Y heated in 80°C; Z heated in 90° C

Summary of the results presented in Table 15

- Seaweed Agarose was essential for gel viscosity (samples 1.2.3);
- Heat treated whey powder increased the viscosity slightly compared to unheated whey powder (samples 4 and 5), although water holding was not improved;
- Heat treated skimmed milk powder significantly increased the viscosity and water holding, compared to unheated skimmed milk powder (samples 6 and 7);
- Heat treated skimmed milk powder significantly increased viscosity and water holding compared to heat treated whey (samples 8 and 9); and
- A combination of 3% heated whey powder and 2% heated skimmed milk was found to reduce agarose concentration to 0.5%, to give gel a high viscosity (sample 8).

These results correspond with results regarding increased viscosity of emulsions made with heat-treated (90^{o}C) camel skimmed milk powder. These results confirm the postulation that controlled heat treatment of camel skimmed milk would improve

viscosity and water holding in acidic emulsions. Preheat treatment of camel skimmed milk result in disulphide bond formation and glycation reactions (chapter 2), which would form stable aggregates. Inclusion of these preformed aggregates into liquid full fat camel milk (Mix B), followed by further heating (45 minutes at 90 C), leads to formation of even larger aggregates, which when acidified, forms larger coagulates with casein and leads to increased entrapment of fat and water, thus forming a harder gel. We report here for the first time (to our knowledge), on the improved viscosity and water binding ability of heat treated skimmed milk powder in acidic milk gels. We decided to use sample 7 composition to develop fermented yoghurts, mainly because of the relative simplicity of the process for preparing freeze-dried powder made of heat treated skimmed milk.

3.3.8 Effect of addition of casein powder on physical properties of acidified GDL gels

A concentration of 4% casein powder was added to camel milk (Milk Mix A, Table 13) and the pH was adjusted to 7, with 1 M NaOH. The milk mixture was further processed according to Process A (as shown Figure 42).

Table 16 Comparison of the properties of gels containing dissolved casein powder. Casein containing gel is used to replace whey protein

Samples	Water separation %	Texture Newton	Viscosity Pas
Non heated camel casein Mix A, Table 13(TS: 15.5%)	0.5±0.3	1.80±0.1	28.11±0.32
Sample 7 Table 15 (pre heated camel milk 90°C) TS : 14.5%	15±0.2	0.94±0.6	27.59±0.1
Commercial yogurt made with Bovine milk	0.5±0.42	0.96±0.08	11.4±0.3

The results show that the additional camel casein significantly reduced syneresis to levels similar to that found in commercial yoghurt, albeit with higher total solids of gel made from preheated skimmed milk powders. Additional casein forms a harder

acidified gel than additional skimmed milk powder. The reason could be that the casein powder contains less lactose hence the total protein content in the gel would be higher and less lactose to weaken the gel network.

The improved water binding ability noted could be due to the camel casein forming a fine gel network, which has a good ability to entrap water,

To the best of our knowledge, this is the first time that the benefits of camel milk casein powder have been demonstrated in acidic milk gels. These are that it is easy to solubilise in milk at pH 7, that it forms a smooth texture with high viscosity, and that it has demonstrably high water binding ability.

3.4 Conclusions

In this chapter, camel milk gels were developed to serve as basis for development of fermented yoghurt. It consisted of:

- Whole camel milk containing 4% pre-heated skimmed milk powder and 1% seaweed agarose, acidified by 4% GDL;
- Whole camel milk containing 4% camel casein powder, and 1% seaweed agarose, acidified by 4% GDL; and
- The optimal heat treatment of the milk mixture before acidification, which was established as 45 minutes at 90 ºC.

The following was demonstrated:

- Camel milk forms lower viscosity gels with smaller coagulates than bovine milk as revealed by confocal microscopy.
- The addition of preheat treated skimmed milk powder (90ºC) significantly enhanced viscosity and reduced syneresis in the acid gel, compared to the non-heat treated control. To our knowledge, this is the first time that this has been reported.
- The addition of preheat treated whey powder (80 ºC) did not significantly enhance viscosity nor reduced syneresis.
- Camel milk casein powder results in increased viscosity, reduced syneresis and smooth textured gels. To our knowledge this is the first time that this has been reported.
- SDS PAGE followed by Coomassie staining revealed formation of proteins with a high molecular weight, by disulphide bonding after 45 and 60 minutes heating of acid gels, corresponding to increased acid gel viscosity.
- SDS PAGE followed by Ponceau staining revealed formation of glycated proteins with a high molecular weight, after 45 and 60 minutes heating of acid gels, corresponding to increased acid gel viscosity.

CHAPTER 4

FERMENTED CAMEL MILK YOGHURT

4.1 Introduction

4.1.1 The Yoghurt market

Bovine milk products fermented with different bacterial cultures have been consumed widely around the world, particularly in Europe. More than 9 million tonnes of yoghurt was produced worldwide in 2001, of which 6.6 million tonnes came from Europe (Sodini, et al., 2007). Yoghurt has also become increasingly popular in regions outside Europe. In particular, the USA yoghurt market has grown rapidly over the past few years, with annual growth rates rising from 3% to 10% (Guinee et al., 1995). Camel milk yoghurt is not yet produced commercially, primarily because camel milk is only available in certain areas, such as Africa and the gulf areas of Asian countries. Additionally, obtaining a firm gel using camel milk without additional additives has been unsuccessful, as explained in the previous chapter.

4.1.2 Yogurt fermentation with microbial cultures

The yogurt fermentation process depends on the proteolytic system brought about by growth of lactic acid bacteria in the milk, which contributes significantly to flavour development in fermented milk products, where these micro-organisms are used as starter cultures. The proteolytic system is composed of proteinases, which initially cleave the milk protein to peptides. (Broadbent and Steele2007). Camel milk that has been fermented with Kefir (drinkable yogurt not spoonable) has been sold commercially in the city of Santa Monica in the USA (Milk, 2015). The only ingredients in this yogurt are the milk and the microbial culture. However, it is not available in most UK and Europe supermarkets. Furthermore, no previous study has investigated the use of Kefir-fermented camel milk to make spoonable yoghurt.

4.1.3 Ready to use Microbial cultures applied in the study

1) Kefir

Guzel-Seydim et al (2000) have reported that Kefir culture consists of several strains of probiotic bacteria, not found in regular yoghurt cultures, and that it also includes a complex mixture of lactic acid bacteria (*Lactobacillus, Lactococcus, Leuconostoc*, and *Streptococcus*) and yeast species (*Kluyveromyces, Saccharomyces*, and *Torula*) woven into a polysaccharide–protein matrix. These yeasts and lactic acid bacteria are associated symbiotically, and are responsible for lactic-alcoholic fermentation (Chia Chen et al., 2008; Zhou et al., 2009). Kefir grains form a complex micro flora, which consists of more than 100 varieties of bacteria and yeasts. *Lactobacillus kefiranofaciens*

is a major bacterial species found in Kefir (Cheirsilp, 2003), along with several unidentified species of *Lactobacillus* (Chen and Chen, 2013). The *Lactobacillus* group of cultures has been utilised in previous attempts at manufacturing camel milk yoghurt. Studies done to date indicate that camel milk yoghurts form a very soft curd, and are less firm and more fragile than those made with bovine milk (Rahman et al., 2009).

***2) L.bulgaricus* and S*treptococcus thermophilus* cultures group**

This freeze-dried starter culture was used in this chapter, not as a monoculture, but as a carefully balanced blend of *lactobacillus bulgaricus* and *streptococcus thermophiles*; the benefit of these microorganisms are well known to promote gastrointestinal health (Awad et al., 2005).

3) Yo Go

The main ingredient of the YO GO microbial culture group is (non-gmo maltodextrin from corn), s. *thermophilus*, *l. bulgaricus*, *l. acidophilus*, and the bacterial strain *Streptococcus thermophilus* which is known to promote gastrointestinal health. *Streptococcus thermophilus*, along with *Lactobacillus bulgaricus*, soon became the starter strains used to make yogurt. Today, these two probiotics continue to be used in the production of true yogurt (Awad et al., 2005).

4.1.4 Aims of this chapter

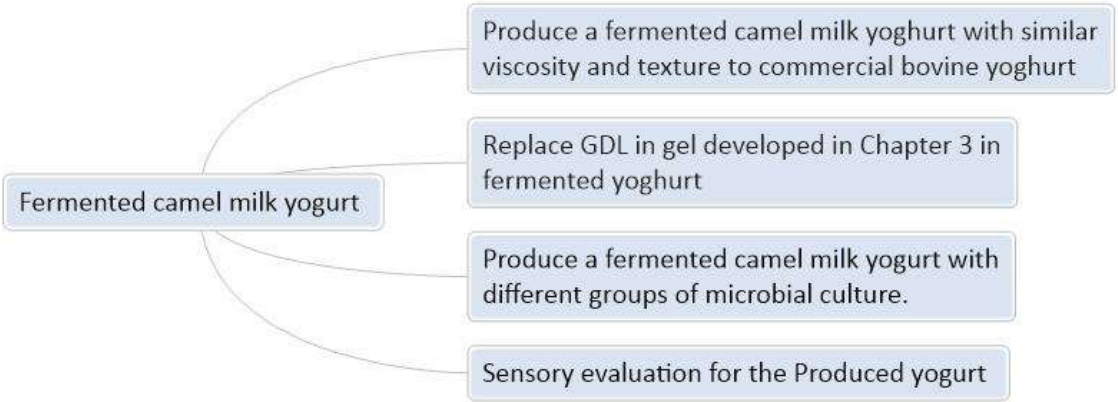

Figure 43 Aims for Chapter 4

4.1.5 Novelty of the study

This study is unique because it is the first time camel milk proteins have been added to increase total solids of camel milk to prepare a fermented spoonable yogurt.

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4.2 Materials and Methods

4.2.1 Materials

4.2.1.1 Food raw materials

Pasteurised camel milk from Saudi Arabia *(al-turath al- Saudi)*, which was transported to Edinburgh in frozen lots.

- Bovine pasteurised skimmed milk was bought from the local supermarket (ASDA Fresh Milk Skimmed).
- Agarose (seaweed gelatine): Ceylon moss (Gelidium amansii) – common name china grass or agar – was obtained from Intralabs, UK.
- Vegetable oil was obtained from the local supermarket (ASDA)

4.2.1.2 Bacterial starter cultures

The raw bacterial starter cultures and yogurt making equipment were as follows:

- Kefir culture (a mixture of bacteria and yeast) obtained from Homestead Farm Supplies, UK.
- (YO GO) Non-Dairy Yogurt Starter *(S.thermophilus, L.bulgaricus, L.acidophilus, and Streptococcus thermophilus) o*btained from Homestead Farm Supplies, UK.
- *L.bulgaricus and Streptococcus thermophilus* obtained from Homestead Farm Supplies, UK

4.2.2 Methods

4.2.2.1 Determination of the best concentration for a starter culture

The microbial cultures were weighed on the sensitive scale, and added to Milk Mix B and processed based on process A (Figure 42 Chapter 3). Different concentrations of starter culture were added to the heat treated milk mixture after cooling at 40ºC. This process was then followed by incubation at 40ºC, until the pH reached 4.3, which is approximately 12 hours. Then samples were stored at 4ºC overnight. Texture, viscosity, pH, and syneresis were also measured.

4.2.2.2 Optimising total solids fermented yoghurt

Using Milk Mix A and B, and Table 13 in Chapter 3, the replacement of 4% GDL was tested using varying concentrations of heat treated camel skimmed milk powder, seaweed agarose, and non-heated camel casein powder.

Casein powder was added to the mix, and 1 molar NaOH was added to adjust the pH to 7 to dissolve the casein completely. Camel milk containing powders were stirred for 30 minutes before following Process A (Figure 42 Chapter 3), except that the microbial culture was added after cooling the heat treated mixture to 40ºC.

4.2.2.3 Measurement of gel and yoghurt properties

4.2.2.3.1 Yogurt syneresis

The syneresis was measured as described in Chapter 3 section 2.2.7

4.2.2.3.2 Determination of hardness of yoghurt texture

Triplicate samples of yoghurts were compressed to a 50% deformation using a Zwick/Roell type Z010 machine Speed test cycles 10mm/min.

4.2.2.3.3 Confocal laser scanning microscopy

As described in Chapter 3 section 3.1.5.2

4.2.2.4 Sensory evaluation

A survey sheet was prepared to record the sensory score. The scores for how satisfactory the results were are expressed as smiley faces at the bottom of the survey. The survey sheet elements were selected according to the most important aspects to be assessed in terms of the yogurt. Each element on the survey sheet was explained verbally. The yogurt colour was evaluated according to panel's desirability. Yogurt Firmness and smoothness acceptability by comparing samples together. Flavours acceptability comparing to each yogurt sample, and the level of sourness. Last parameter was measured the general acceptability as a new product.

Panels were chosen randomly from the researcher's university friends, and the total number of completed surveys was 20, where all question were answered clearly.

All the completed survey sheets data was combined into one excel sheet. There was no control sample for comparison. Sensory evaluation analyses were performed according with some modification.

Table 17 Sensory evaluation parameters

Samples	Colour %	Firmness %	Smoothness %	Test %	Flavour %	Sourness %	Acceptability %
8% camel casein + 2mg/100ml yogurt culture + 1% seaweed agarose							
1	Kefir						
2	*L.bulgaricus + S.thermophilus*						
3	Yo Go						
8% heat treated camel skimmed milk + 2mg/100ml yogurt culture + 1% seaweed agarose							
4	Kefir						
5	*L.bulgaricus + S.thermophilus*						
6	Yo go						

Table 18 The survey sheet that was used for the sensory evaluation

Samples	Colour	Firmness	Smoothness	Test	Flavour	Sourness	Acceptability
Group 1							
1							
2							
3							
Group 2							
4							
5							
6							

1 2 3 4 5 6 7

4.3 Results and discussion

4.3.1 Determination of the best yogurt culture concentration after 13 hr fermentation

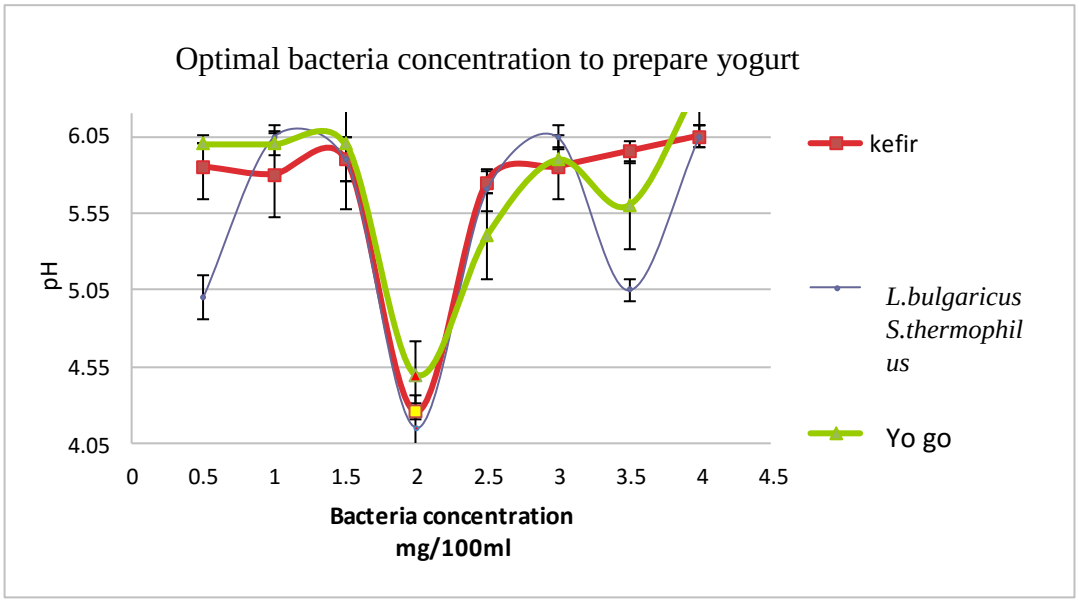


Figure 44 The effect of culture type and concentration on pH of milk Mix B

The results revealed that the best concentration for the microbial cultures was 2mg/100ml for each.

The lower and higher concentration of bacteria comparing to 2mg/ml was it insufficient to ferment the yogurt in the desirable pH. Low concertation of bacteria Figure 44 shows very low lactic acid produced by bacteria that can be demonstrated with stable pH with 0.5,1 and 1.5 mg/ml. Moreover, that could be due to the antimicrobials activity against the microbial cultures, which they stable in high temperate. Fermented camel milk with high concertation of bacteria 2.5, 3, 3.5,4 and 4.5 mg/ml shows similar activity with < 2mg/ml bacteria, that could be due to cloning very high numbers of bacteria, and that make it not possible for bacteria to produce lactic acid (Pawar et al., 2000).

4.3.2 Optimisation of total solids

In the previous chapter, the Mixes A and B (Table 13) contained 4% GDL. In order to replace the total solids for GDL in fermented yoghurts, the total solids had to be readjusted. The aim of this step was to adjust the texture with increased/reduced total solids with camel casein powder, skimmed milk powder, or seaweed agarose powder.

Fermentation was with 2mg/100ml of Lactic acid (*L.bulgaricus + S. Thermophilus*) a common starter culture used for bovine milk yoghurt .

Table 19 Texture, viscosity, and water separation of yogurts prepared with microbial culture

Sample	Agarose %	Casein %	Preheated SMP%	Syneresis %	Texture Newton	Total solid %	Viscosity Pas
1	4	0	5	None	3.06±0.4	15.5	too high
2	3	0	6	10±0.12	0.53±0.2	15.5	80.356±0.68
3	2	0	7	15±0.32	0.36±0.3	15.5	68.705±0.5
4	1	0	8	20±0.19	0.39 ±0.5	15.5	16.96±0.2
5	1	8	0	10±0.5	1.01±0.8	15.5	19.651±0.13
C				0.5±0.42	0.96±0.5		11.4±0.3

C= commercial yogurt

The results illustrated in Table 19 demonstrate that the ideal concentration of agarose to be applied with yogurt was 1% (according to Table 15). The results of the hardness and viscosity parameters for sample 4 in Table 19 indicated the nearest value to that of the commercial yogurt. It was prepared with 1% agarose and 8% pre-heated camel milk powder at 90^{o}C, and sample 5 (8% camel casein powder, 1% agarose) .

The camel casein sample 5 in the Table 19 shows significant improvements in water separation compared to sample 4 (8% preheated skimmed camel milk powder) in the 90^{o}C (sample 4): 10 ±0.5 versus 20 ±0.19, Furthermore, viscosity and texture were also increased.

Table 20 Ingredients for two types of yoghurts for fermentation with microbial cultures.

Group	Ingredients
Camel milk	8% camel skimmed milk powder pre-heated at 90°C, 2mg/100ml yogurt starter culture, 1% seaweed (agarose) powder, and 92% camel milk (liquid form)
Camel casein	8% non-heated casein powder, 2mg/100ml yogurt starter culture, 1% seaweed (agarose) powder, and 92% camel milk (liquid form)

4.3.3 Camel milk yogurt with three different types of yogurt cultures

Samples containing: preheated skimmed camel milk powder, or camel casein powder Table 20 were prepared with 3 different types of yoghurt cultures. The purpose of this step was to compare the sensory and physical properties of the results, such as texture and syneresis.

4.3.3.1 Yoghurt containing preheated skimmed milk powder (8%)

Table 21 The properties of yoghurt with skimmed milk powder fermented with 2mg/100ml different starting cultures

Yogurt culture	Viscosity (Pa)	Texture Newton	Total solid	Syneresis%
1	13.01±0.78	0.45±0.27	15.5	20±0.5
2	16.96±0.2	0.39±0.51	15.5	20±0.75
3	18 .09±0.92	0.89±0.23	15.5	20±0.5
C	11.4±0.3	0.96±05		0.5±0.42

C= commercial yogurt

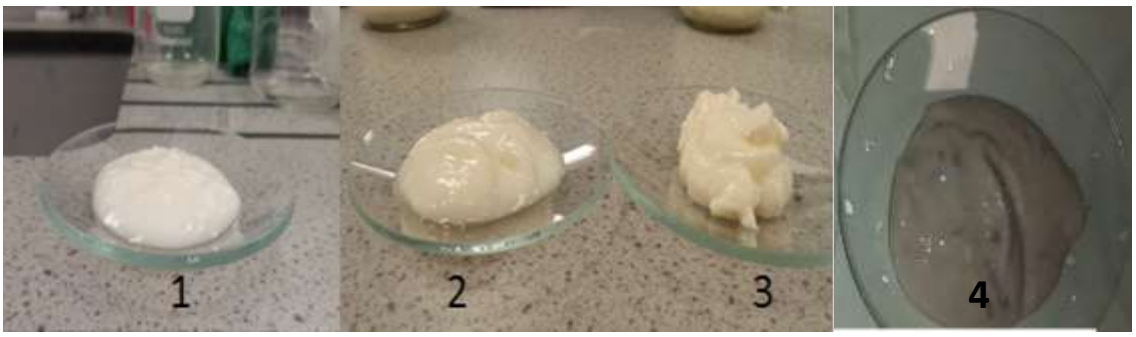


Figure 45 The properties of camel skimmed milk (8%) yogurts, prepared with different microbial cultures. 1) Kefir 2) *L.bulgaricus + S. thermophiles* 3) Yo go 4) commercial yogurt

4.3.3.2 Yoghurt prepared with Camel casein powder (8%)

Table 22 The properties of yoghurt containing camel casein fermented with different starting cultures

Yogurt culture	Viscosity Pas	Texture Newton	Total solids %	Syneresis%
1	15.92±0.87	0.81±0.74	15.5	10
2	10.43±0.33	0.22±0.89	15.5	10
3	18.90±0.79	0.55±0.75	15.5	0
C	11.4±0.3	0.96±0.5		0.5±0.42

C= commercial yogurt

Figure 46 The properties of camel casein (8%) yogurts, prepared with different microbial cultures. 1) Kefir 2) *L.bulgaricus + S. thermophiles* 3) Yo go 4) commercial yogurt

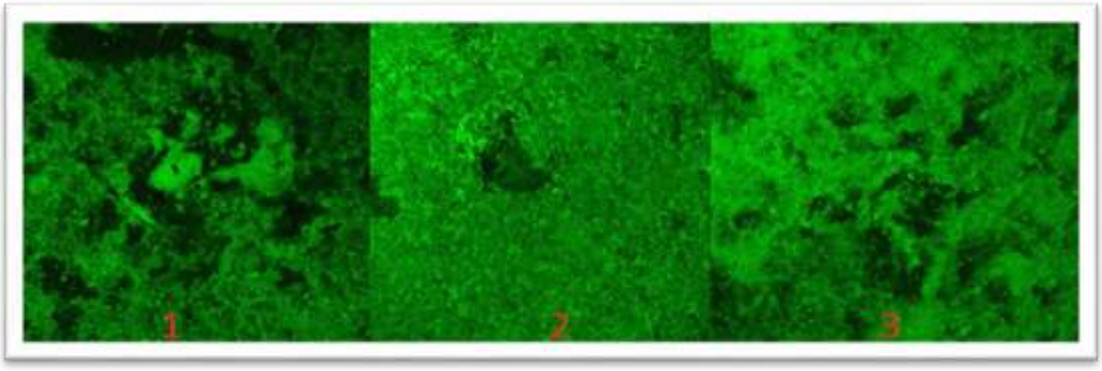


Figure 47 Confocal microscopy of Camel milk yogurts (containing 8% casein) prepared with three different cultures: 1. Kefir; 2. *L.bulgaricus + S.thermophiles*; and 3. Yo Go).

Sample 2 Figure 47 shows fine texture of yoghurt fermented with *L.bulgaricus + S.thermophiles* compare to the other two other bacterial cultures (sample 1 and 3).

Which correspond to its lower viscosity and hardness. Figure 47 shows that milk gel fermented with L.bulgaricus + S.thermophiles was provided soft gel comparing to the samples 1and 2. Commercial yogurt in Figure 46 sample 4 shows the softest gel sample comparing to camel milk yogurts (all three samples) that could be due to yogurt homogenised after fermentation process. Sample 2 viscosity was the closest to the commercial yogurt, but the texture was significantly low. The optimal properties of came milk yogurt was obtained with sample 1, which was fermented with Kefir microbial culture.

In this chapter, skimmed milk powder or casein powder were combined with 1% agarose to improve the coagulation and textural properties of the yogurt. The novelty of the application was that, it is first time camel milk yogurt was developed with 1% agarose and 8% camel casein powder.

Fermentation of camel milk with microbial cultures has proved difficult, largely because of the high content of antimicrobial compounds in camel milk. A study by El Agamy et al. (1992) explored the antimicrobial activity, which is represented in Lysozyme (LZ), lactoferrin (LF), lactoperoxidase (LP), immunoglobulin G, and secretory immunoglobulin, and which characterised camel milk as having a very high defence against microbes. The camel milk LP was bacteriostatic against gram-positive strains, including lactobacillus. These elements are considerably resistant to high heat, especially camel milk lysozyme (Elagamy, 2000), which appears to demonstrate activity against gram-positive bacteria (Mekkaoui et al., 2004). In conclusion, previous studies indicate anti-microbial activity could affect the growth of bacteria casing camel milk to be resistant to fermentation. Here we have demonstrated fermentation of camel milk with additional camel proteins and agarose to obtain a set yoghurts with viscosity and syneresis similar to commercial yoghurt.

4.3.4 Sensory evaluation of camel milk yogurt prepared with three different starter cultures

Photographs of the samples prepared for sensory evaluation. The composition of each is described in Table 23. Six types of yogurts were made with camel skimmed milk powder milk (samples 1, 2, 3); and samples 4, 5 and 6 were made with camel casein powder.

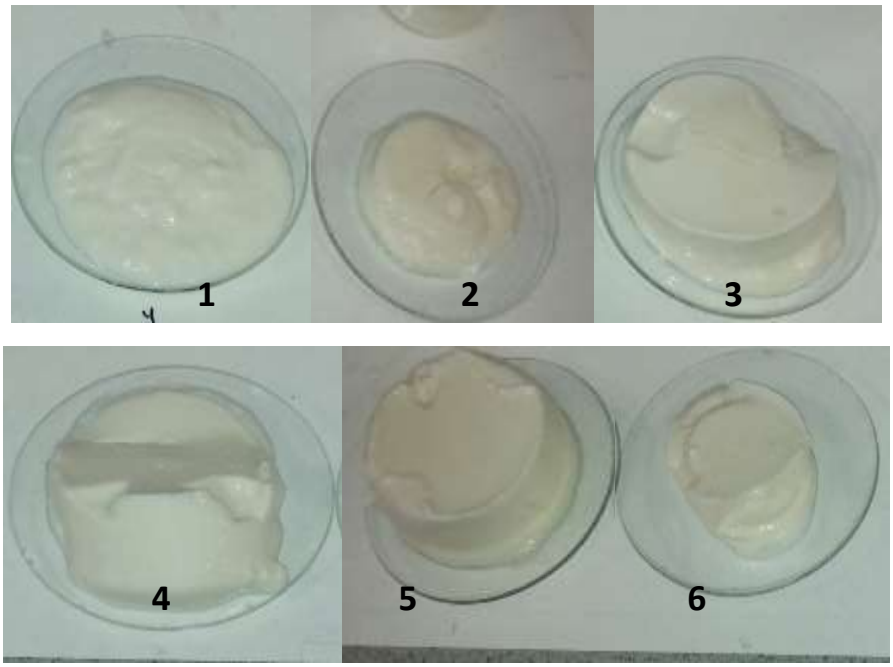


Figure 48 The texture and colour of the yogurts prepared for sensory evaluation

Table 23 Shows the panel's sensory evaluation results for each parameter (Values are given as %)

Acceptability	Colour	Firmness	Smoothness	Mouthfeel	Flavour	Sourness	Culture
8% camel casein + 2mg/100ml yogurt culture + 1% seaweed agarose							
82.5	78.5	77	76.5	65.5	60.5	68.5	1
54	76	60.5	69.5	48	54.5	62.5	2
25.5	59.5	62.5	64.5	30.5	37	55.5	3
8% camel milk + 2mg/100ml yogurt culture + 1% seaweed agarose							
81	83.5	69.5	72.5	64	52	60	4
62	84.5	58.5	41	62.5	58	55.5	5
21	35	75	58.5	51.1	28.5	51.5	6

The results of sensory evaluation are shown in Table 23. The samples had to be compared with each other, because there is no commercial camel milk yogurt available for use as a control sample. Yogurt prepared with 8% camel milk, pre-heated to 90^{o}C was compared with yogurt made with 8% non -heated casein. Significant differences were observed in **Error! Reference source not found.**between the mean values of the groups. The results highlighted in Figure 55 and confirm that the yogurt made with

Kefir culture was the most desirable sample for both camel casein and milk. The differences between the samples were compared with each other to show the panel's desirability ratings.

4.3.4.1 Colour

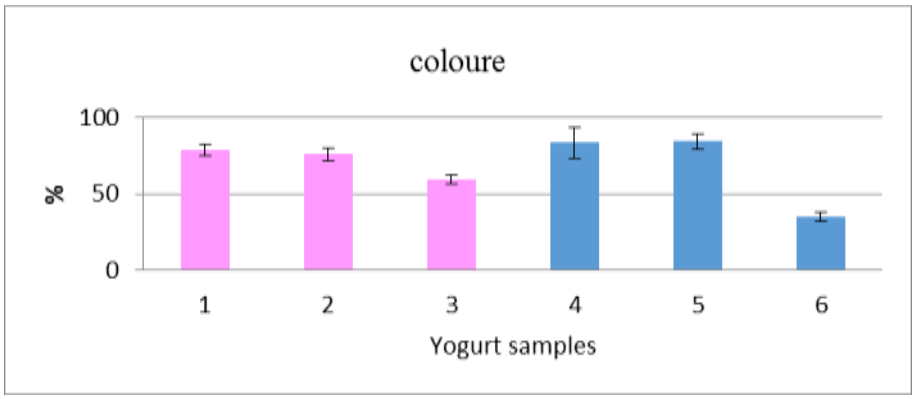


Figure 49 The evaluation of the colour of yogurts made with the three different starter cultures.

The colour of each yogurt was white; however, the level of whiteness varied slightly between each yogurt, and some were found to be more desirable than others. The two most desirable colours were samples 1and 2 in camel milk group and 4 and 5 with camel casein samples. Sample 3 shows the lowest desirable microbial culture in terms of colour and appearance. In conclusion, camel milk yogurt samples 1 and 2, which were fermented with Kefir and *L.bulgaricus* + *S.Thermophilus*, respectively, were found to be the most similar to commercial yogurt colour.

4.3.4.2.2 Firmness

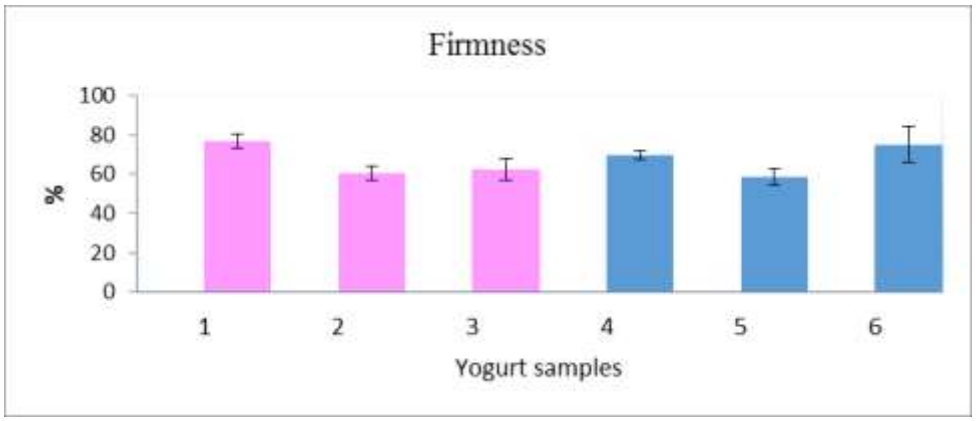


Figure 50 The firmness evaluation for the yogurts made with three different starter cultures.

The firmness of the yogurt was measured by how it was accepted by panels.

Figure 50 demonstrates that the firmness of the yogurt did not differ significantly between each example. However, the most preferable firmness was observed with sample 1 in camel casein, which fermented with Kefir. Moreover, sample 6 from camel milk yogurt, which was fermented with YO GO. The sample details in Figure 50

Confirm the firmness results.

4.3.4.2.3 Smoothness

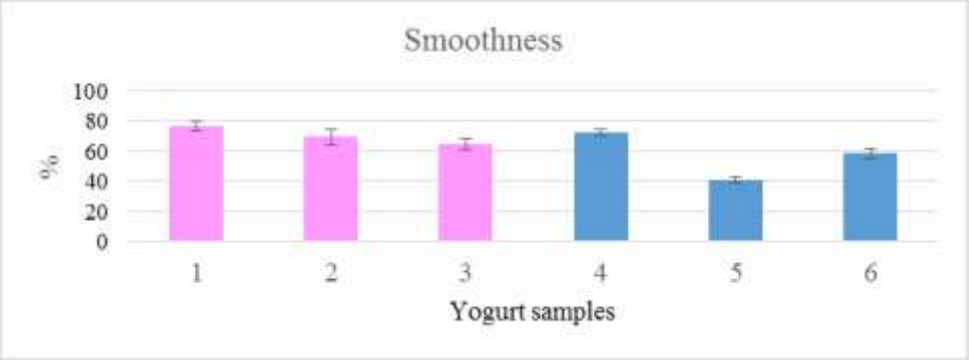


Figure 51 The smoothness evaluation for yogurts made with three different starter cultures.

The smoothness of the yogurt was evaluated to support a comparison between typical yogurt smoothness. The results indicated that the yogurt with the most preferable smoothness was sample 1, in the case of both camel casein and camel milk yogurt. In contrast, a less desirable smoothness was observed with sample 5, camel milk, which was prepared with *L.Bulgaricus* + *S.Thermophilus*. In conclusion, yogurt smoothness was observed to be most preferable with sample 1 then 4 in camel milk and casein yogurts respectively, although it was not significantly different, when fermented with the Kefir microbial culture

4.3.4.2.4 Mouthfeel test

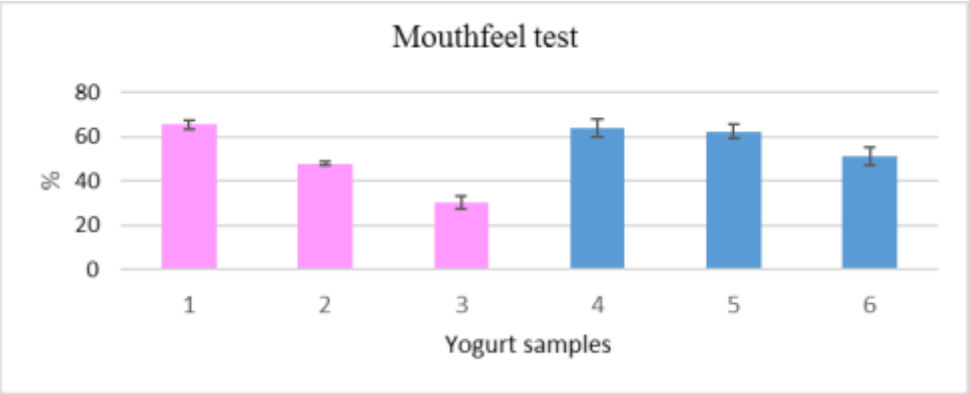


Figure 52 The mouthfeel test evaluation for yogurts made with three different starter cultures

The purpose for considering this parameter was to explore the acceptability of the yogurts in terms of the way they feel in the mouth.

Panels evaluated the test properties of all the yogurts, and indicated their preferences. Consequently, sample 1 for camel casein and camel milk, was prepared with the Kefir microbial culture. In contrast, the less desirable yogurt was sample 3, camel casein, prepared with YO GO, as shown in Figure 52. In conclusion, samples 1 and 2 camel casein yogurt, and sample 1 camel casein yogurt, were the most desirable samples, as judged by the panels.

4.3.4.2.5 Flavour

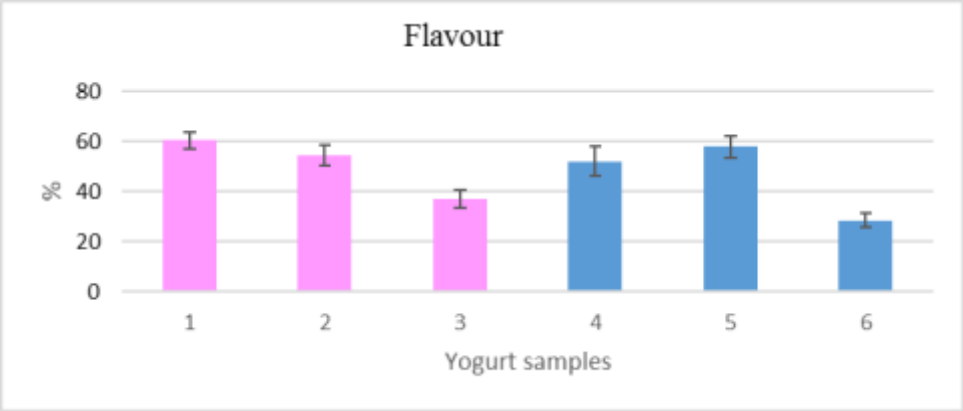


Figure 53 The flavour evaluation for yogurts made with three different starter cultures

Figure 53 illustrates that, sample 1 camel casein, and sample 5 camel milk, were the most desirable yogurt. In contrast, a less desirable yogurt flavour was observed with samples 3 and 6 , camel milk yogurt, which was prepared with a YO GO microbial culture. In conclusion, the most desirable yogurt flavour was observed with camel casein samples that had been fermented with Kefir microbial culture. Camel milk was fermented with L.bulgaricus + S.Thermophilus. Kefir microbial culture was not as preferable with camel skimmed milk as it was with camel casein yogurt samples. As well as YO GO, a microbial culture was not desirable for both camel and casein yogurts.

4.3.4.2.6 Sourness

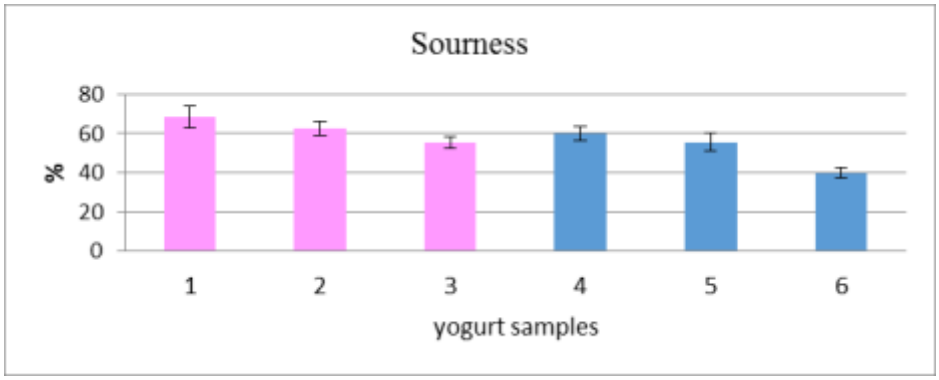


Figure 54 The sourness evaluation for yogurts made with three different starter cultures.

As it shows in figure 54 no significant difference was reported between the samples. However, the camel casein yogurt, sample 1, was characterised by having the most desirable sourness.

4.3.4.2.7 General acceptability

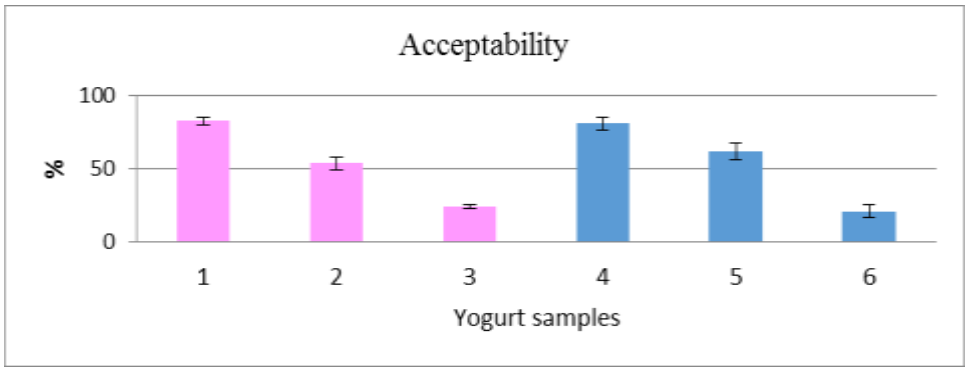


Figure 55 The acceptability evaluation for yogurts made with three different starter cultures.

The aim of this parameter was to measure the general level of acceptability. The results revealed that desirability gradually decreased from samples 1 to 6 for both yogurt groups. In general, sample 1, camel casein, and sample 4, camel milk, were the most preferred yogurt samples.

In conclusion, the samples could be divided into three groups, dependent on microbial culture. The most desirable microbial culture was Kefir, then *L.bulgaricus* + *S.Thermophilus*, and finally YO GO, in both camel milk and casein fermentation cases.

4.4 Conclusion

The process for preparing spoonable fermented yoghurt is outlined below

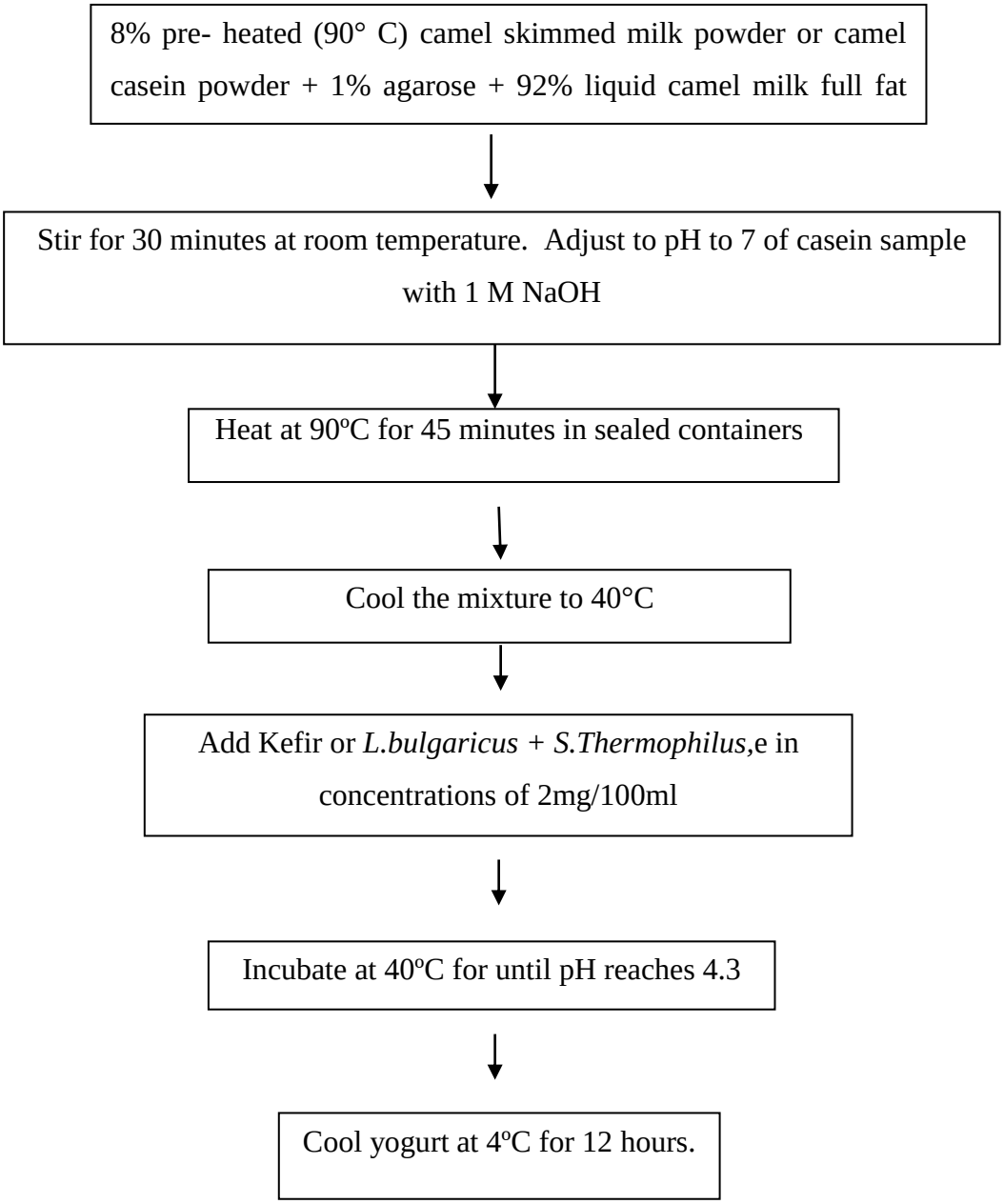


Figure 56 Process for preparing spoonable fermented yogurt from camel milk

Camel casein yogurt, prepared with Kefir and *L.bulgaricus* + *S.Thermophilus* provided the best texture, viscosity, pH and syneresis for both camel milk and casein samples when compared to the YO GO microbial culture.

Results of sensory evaluation showed that the most desirable sample was camel milk and casein yogurt made with Kefir. Whereas, the least desirable sample, was made with

the YO GO microbial cultures. In addition, yogurt prepared with casein reported the highest desirability compared to yogurt made with camel milk when using the same microbial culture.

There are many published camel milk yogurt projects (Hashim, Khalil and Habib, 2009; Abu-Tarboush, 1996; Edrees, 2013). However, that done by Khalifa and Ibrahim (2015) has the most similar ingredients to those used in this project. Furthermore, these ingredients were the easiest to obtain commercially. The authors prepared camel milk yoghurt by the addition of bovine gelatine E441, mono and diglycerides of fatty acid E471; guar gum E412, sodium carboxymethyl cellulose E466, modified starch E1422, mono and triglyceride of fatty acid E471 to fresh camel milk, to provide high viscosity and prevent serum separation In this chapter, seaweed agarose only was applied as a yogurt stabilizer. As the remainder of ingredients were camel milk fractions (skimmed milk or casein powder), thereby recducing the number of E-number ingredients

CHAPTER 5

CHARACTERISATION OF PROTEIN WITH INSULIN-LIKE ACTIVITY IN CAMEL MILK

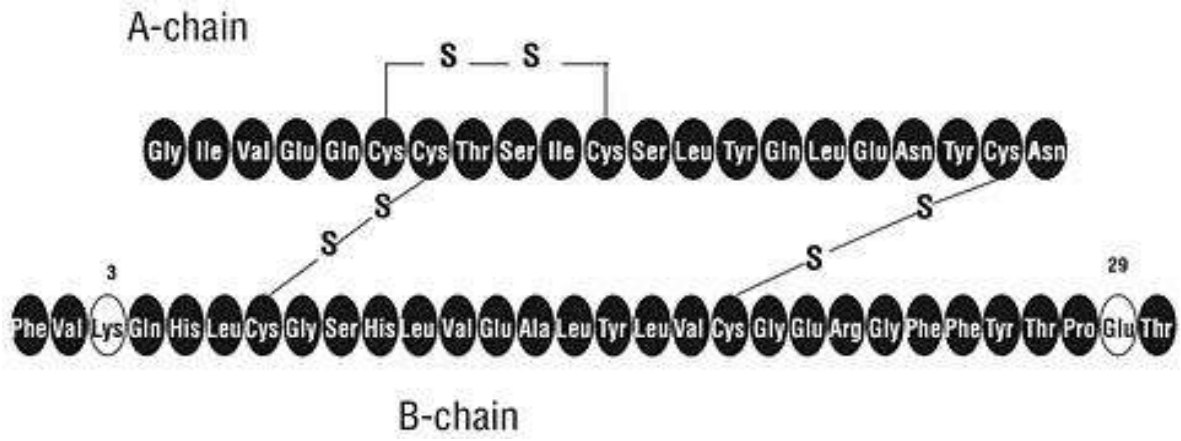
5.1 Introduction

5.1.1 General introduction

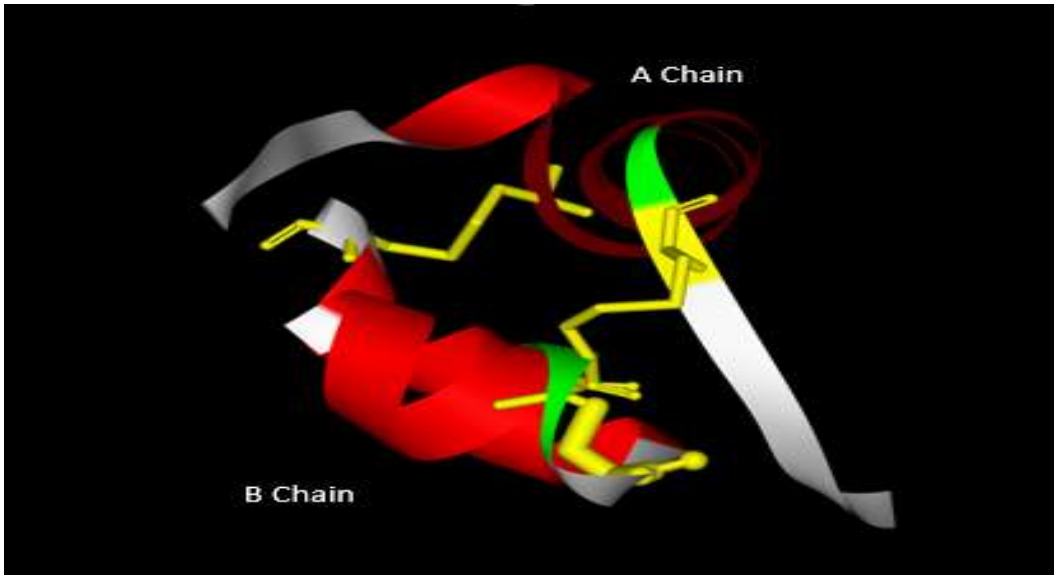
The number of diabetic patients in Saudi Arabia is expected to reach an estimated 5,462 million by 2030 (Whiting et al., 2011), at a likely cost to the country of up to US $490 billion (Almutairi & Alkharfy, 2013). Control et al. (2011) found that the risk of death among individuals with diabetes is approximately twice that of those of a similar age who do not have diabetes. Furthermore, medical expenses for people with diabetes are more than twice as high as for people without diabetes. Diabetes is thus a serious, chronic problem in Saudi Arabia, and one for which natural cheap treatments are preferable to insulin injections. The main objective of this study is to improve the detection of insulin or insulin-like proteins in camel milk, as this knowledge will help the development of a natural and cheap treatment for diabetes. It has been suggested that the anti-diabetic effect reported after consumption of camel milk could be attributed to 'insulin-like' small molecular substances that mimic the interaction between insulin and its receptor (Malik et al., 2012; Agrawal et al., 2004; Agrawal et al., 2005; El-Said et al., 2010; Jankun, 2012). The main objective of this study is to improve the detection of insulin or insulin-like proteins in camel milk, as this knowledge will help the development of a natural and cheap treatment for diabetes.

In mammals insulin is produced by islets of Langerhans in the pancreas. The structure of insulin varies slightly between species of animals; both porcine and bovine insulin are similar to human insulin, but porcine, which comes from pigs, insulin resembles it more closely (Dean & McEntyre, 2004). Insulin is composed of two chains of amino acids, chain A (21 amino acids) and chain B (30 amino acids), which are linked by two disulfide bridges. There is a third disulfide bridge within the A chain that links the sixth and eleventh residues of the A chain together. In most species, the length and amino acid compositions of chains A and B are similar, and the positions of the three disulfide bonds are highly conserved

For this reason, pig insulin can be used to replace deficient human insulin levels in diabetes patients. Today, porcine insulin has largely been replaced by mass-produced human proinsulin using bacteria (recombinant insulin), which has a molecular weight of 5808 Da (Dean & McEntyre, 2004).



The primary structure of insulin (Aventis, 2015)



Tertiary structure of insulin**:** Red = alpha helix, green = beta turn, grey = random coil, yellow = disulphide bonds

Figure 57 Insulin structures

Insulin has several broad actions, including cells in the liver, muscle, and fat tissue to take up glucose from the blood (Figure 58) and convert it to glycogen that can be stored in the liver and muscles. Insulin also prevents the utilisation of fat as an energy source, by up-taking glucose first. In the absence of glucose, or in conditions where glucose is low, insulin stimulates the uptake of stored fat in cells (glycogen). Insulin also has several other anabolic effects throughout the body (Mandal, 2010).

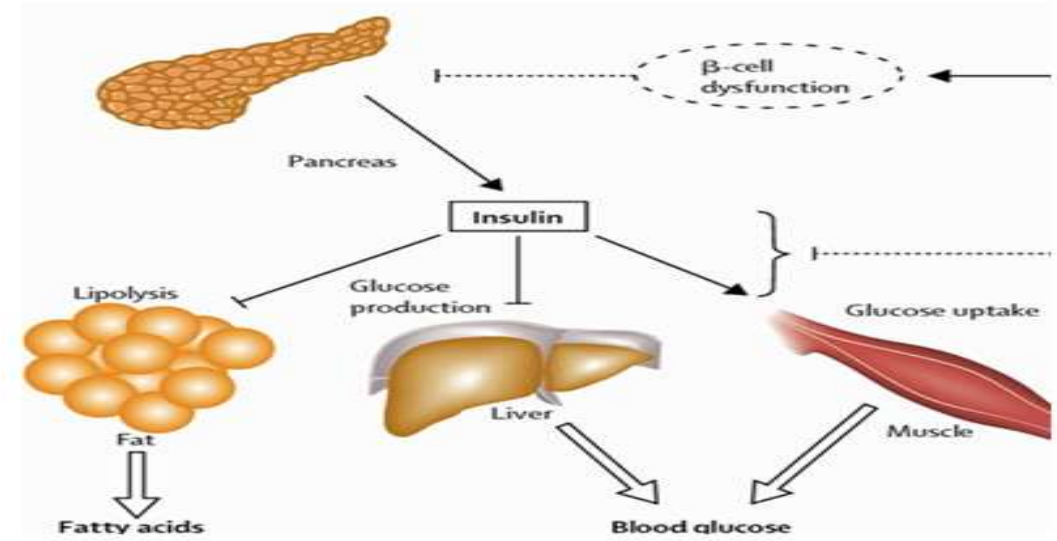


Figure 58 Insulin release from β cells to the muscle, liver and fat cells to stimulate uptake of glucose (Mandal, 2010).

Insulin is only synthesised in significant quantities in β cells found in the pancreas Figure 58 It is secreted primarily in response to elevated blood concentrations of glucose and can regulate blood glucose through this secretion (Zahn et al., 2002).

As insulin controls the central metabolic processes, failure in insulin production leads to a condition called diabetes mellitus. There are two major types of diabetes: type 1 and type 2. Type 1 diabetes occurs when there is no, or very low, production of insulin in the pancreatic beta cells. Patients with type 1 diabetes mellitus depend on external insulin, most commonly injected subcutaneously, for their survival. In type 2 diabetes mellitus the demands of insulin are not satisfied by what is produced by the pancreatic beta cells; this is known as insulin resistance, or 'relative' insulin deficiency. These patients can be treated with drugs to reduce their blood sugar level, or may eventually require externally supplied insulin if other medications fail to control blood glucose levels adequately (Mandal, 2010).

Mammalian milk contains various peptides, such as hormones and growth factors, in addition to nutritive proteins. Some peptides in milk, for instance prolactin and insulin, have been shown to be selectively transported across the intestinal epithelium, (Gonnella, Harmatz & Walker, 1989; Siminoski et al., 1986). Kinouchi et al., 1998 investigated the localisation of insulin in bovine milk. For this study, insulin concentrations were measured in bovine milk that had been subject to ultrafiltration using a 10 KDa molecular weight cut off point membrane, aiming to concentrate the

insulin based on its small size (5.7 KDa). No insulin was found in the UF permeate; these results support the findings of Aranda et al. (1991), who show that insulin is associated with casein. The higher proportion of insulin in the casein fraction indicates that, as a hydrophobic molecule, insulin might interact with milk proteins such as β-casein (Aranda et al., 1991; Ollikainen et al., 2012). Aranda et al., (1991) investigated the insulin concentration in colostrum from Holstein bovines using an ELISA assay. The results showed that at 12 hours after birth the amount in colostrum exceeded 300ng/ml. The concentration of insulin in colostrum was far higher than in blood; bovines must thus have a system to transport insulin from the blood to colostrum (Quigley, 2001).

5.1.2 Evidence for biological activity of insulin in camel milk

The biological activity of insulin in camel milk was investigated by Agrawal (2003) in a study carried out with the Raica community of North West Rajstan, who consume camel milk habitually. The study was designed to measure the blood glucose levels of individuals with a known family history of diabetes. A non-Raica community (i.e., non-consumption of camel milk) negative sample was used. The results show that there was a zero percentage of diabetics within the Raica community suggesting that regular consumption of camel milk is associated with a decrease in prevalence of diabetes (Agrawal, 2003).

Diabetic nephropathy is originally microvascular in nature and is widely considered an important complication of diabetes. Agrawal et al. (2009) carried out a study to determine the efficacy of camel milk in controlling diabetic nephropathy, in which twenty-four type-1 diabetic patients were randomly recruited from the outpatient diabetic clinic in PBM Hospital, Bikaner, India. Urine microalbumin and blood sugar was measured twice a week, before breakfast and dinner. The end of trial results reported a significant improvement in the microalbuminuria after receiving camel milk for 6 months. A significant reduction in the mean dose of insulin for obtaining glycaemic control was also achieved. The therapeutic effect of camel milk on liver and kidney function in diabetic rats was investigated by Romeih et al. (2011). This study was conducted with three groups of 30 diabetic rats, where each group was treated with a different type of milk: bovine, camel, and buffalo milk. The blood glucose levels of the three groups were then measured. At the end of the experimental period, the rats were killed by decapitation, and blood samples were collected from each rat. The results

report a reduction in blood glucose level by ~ 49% for camel milk, compared with 11.6% and 11.1% for the bovine and buffalo groups, respectively.

Agrawal et al. (2011) also investigated the insulin biological activity *in vivo* of camel milk with type-1 diabetic patients. The study examined 24 type-1 diabetic patients over 2 years, where blood glucose was measured twice a week, before breakfast and before dinner, and blood sugar concentration was measured using the glucose oxidase method. Patients were divided into two subgroups; group 1 patients (N=12) received their usual care, diet, exercise and insulin, and the group 2 patients (N=12) received 500 ml of camel milk in addition to their usual care, for three months. The result show that the camel milk group reported a significant reduction in mean blood glucose. Ejtahed et al. (2014) investigated the effect of camel milk consumption on insulin levels in the blood of 20 patients with type 2 diabetes, who were divided into two groups. Participants consumed 500 ml of either camel milk, for the intervention group, or bovine milk, for the control group, daily for two months. Insulin concentration in the blood was measured using the ELISA method, with Mercodia kits (Uppsala, Sweden). The results show that the mean insulin concentration significantly increased from 64.59 to 84.03 pmol/L among the camel milk group. The results conclude that camel milk increases insulin level in patients with type 2 diabetes, and may contribute to glycaemic control for type 2 diabetes (Ejtahed et al., 2014).

Anti-human insulin is the most common antibody applied to detect insulin in milk (Shehadeh et al., 2003),

5.1.3 Evidence for physical presence of insulin in camel milk

Agrawal et al. (2005) measured the concentration of insulin in camel and bovine milk using radioimmunoassay systems an anti-human insulin antibody. The amount of insulin present in human, camel, and bovine milk was found to be 2.7 µg/ml, 1.7 µg/ml and 0.5 µg/ml, respectively.

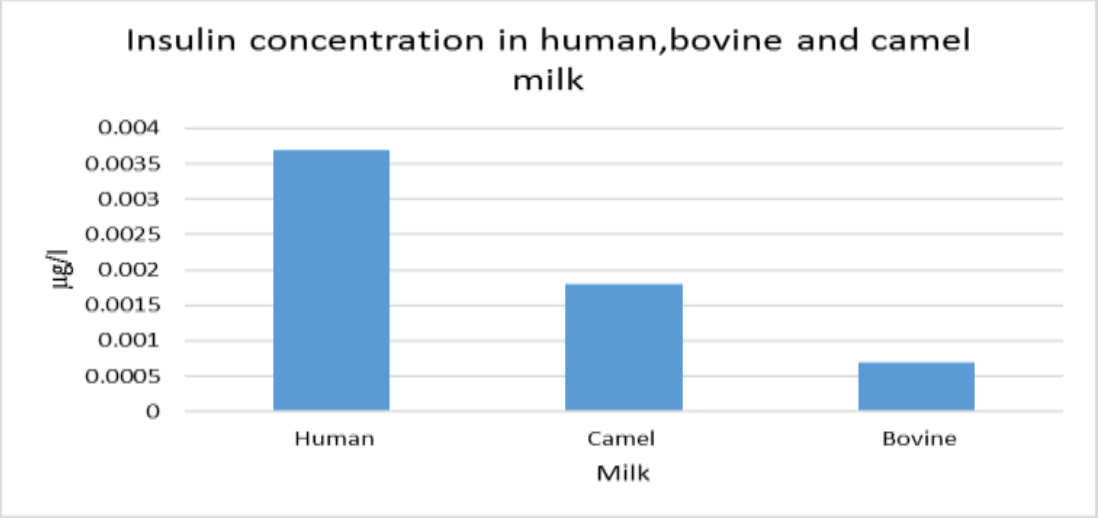


Figure 59 Insulin concentration in three different types of milk (Agrawal et al., 2005), as measured by a radio-immune assay using anti human insulin antibody.

5.1.4 Camel insulin extracted from pancreas

Elamin et al. (2014), note that the molecular size of camel insulin in camel pancreas is 5,800 Daltons, which is similar to that known figures for other species.

5.2 Aims and novelty of this study

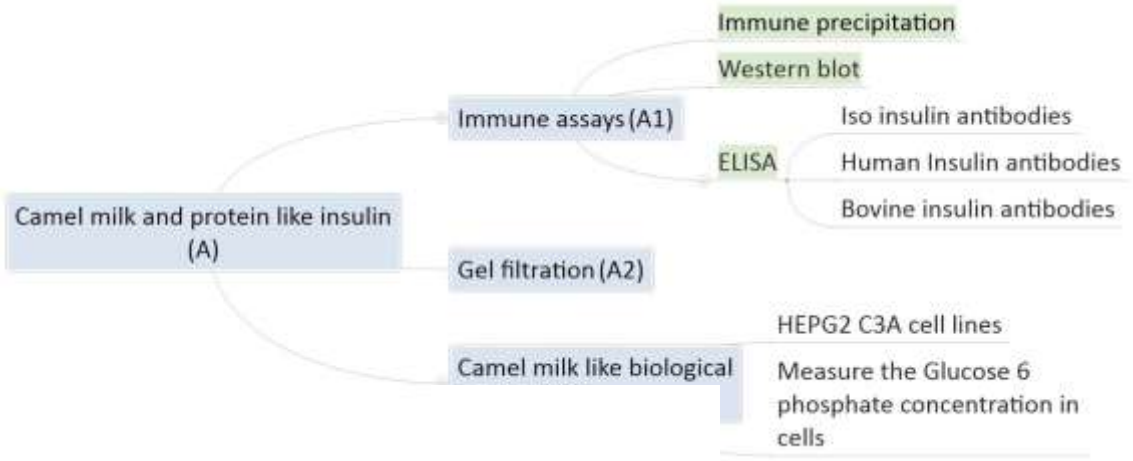


Figure 60 The aims of chapter 5

The aims of this study are to characterise the physicochemical properties of insulin in camel milk through:

- Determination of the concentration of insulin and insulin-like components using immunochemical methods (ELISA) and anti-insulin antibodies of human or bovine origin.
- Localisation of insulin in the whey or casein fraction of camel milk using immunochemical methods.
- Determination of molecular weight of camel milk insulin through immuno blotting.
- Measurement of insulin biological activity using an *in vitro* method.

5.2.1 Novelty of this study

The only methods to date to detecting the physical presence of insulin in camel milk, are based on radioimmunoassay. In this study, the ELISA will be used for the first time, using bovine insulin and human iso insulin antibodies. Furthermore, immune precipitation using protein A and Western blotting with anti-bovine insulin antibody will be conducted for the first time. Determination of biological activity *in vitro*, by the measurement of the activity with hepatic cell lines will be conducted for the first time.

5.3 The methods used in this study to characterise insulin in camel milk

5.3.1 Tests to characterise the physical presence of insulin

ELISA and radioimmunoassay validity has been widely used in previous research to detect insulin in bovine and human milk (Aranda et al., 1991; Read et al., 1984; Whitmore et al., 2012; Shehadeh et al., 2003). The immunoassay is a technique that incorporates the binding reaction of a target substance (antigen) with an antibody. Antibodies are immunoglobins that bind to different natural and synthetic antigens in the body, which are usually proteins with different epitopes. The antibodies have a common structure, but have different special components that help them identify and bind to specific antigens (Hicks, 1984). This specificity of antibodies depends on how many binding sites it has against the target antigen (polyclonal), or just one binding site to a particular antigen (monoclonal).

5.3.1.1 The enzyme-linked immunosorbent assay ELISA

The fundamental principle of the ELISA method is that the target of the analysis (the antigen) is recognised with high specificity by antibodies, which are proteins produced by the immune system. These antibodies (polyclonal or monoclonal) can recognise and bind to the antigens, and the labelling of the bound antibody forms the basis of the detection (Figure 61; Thompson, 2010).

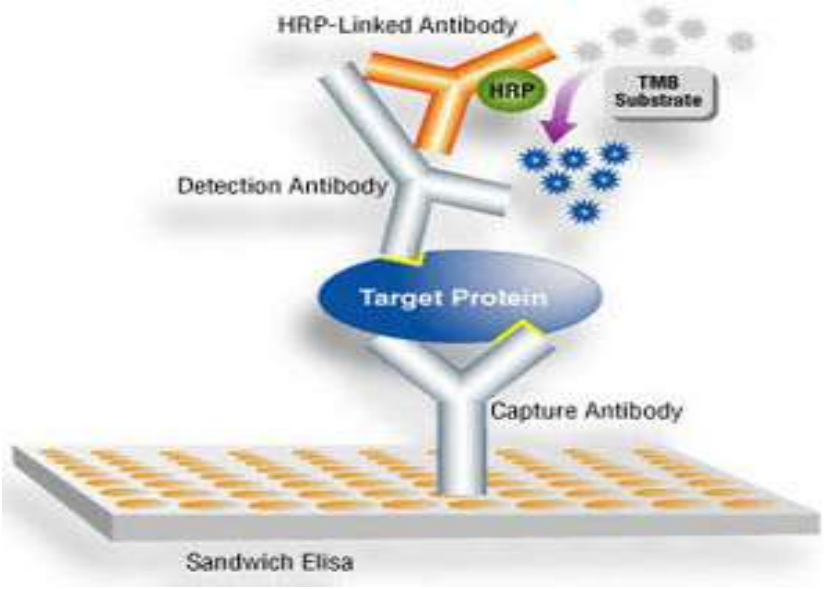


Figure 61 Schematic diagram of the ELISA technique (Affilogic.com, 2015).

The validity of the ELISA assay in measuring the insulin family in bovine and human milk is examined by Guidi et al. (2001), Ginjala and Pakkanen, 1998; McGrath et al. (2008), Ahuja (2011) and Whitmore et al. (2012).

The principle of antibody procedures used in this study

Mercodia Insulin ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique, in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation, insulin in the sample reacts with mouse anti-human insulin antibodies bound to microplate wells. The wells have incubated with camel milk, and the insulin reacts with the antibody coated on the plate. The plate was washed, and incubated with a second monoclonal mouse anti human insulin antibody (the detection antibody). The plate was washed once more, and incubated with HRP labelled rabbit anti mouse IgG. A simple washing step removes unbound enzyme labelled antibodies. The bound conjugate is detected through reaction with 3,3',5,5'-tetramethylbenzidine (TMB). The reaction stopped by adding acid to give a colorimetric endpoint that was read it spectrophotometrically

5.3.1.1.1 Polyclonal and monoclonal antibodies

There are two major types of antibodies, polyclonal and monoclonal, as shown in
. The decision regarding whether to use polyclonal (PAbs) or monoclonal (MAbs) antibodies depends on a number of factors, the most important of which are its intended use and whether the antibody is readily available from commercial suppliers or researchers. The principal advantages of MAbs are their homogeneity and consistency (Pavlou & Belsey, 2005).

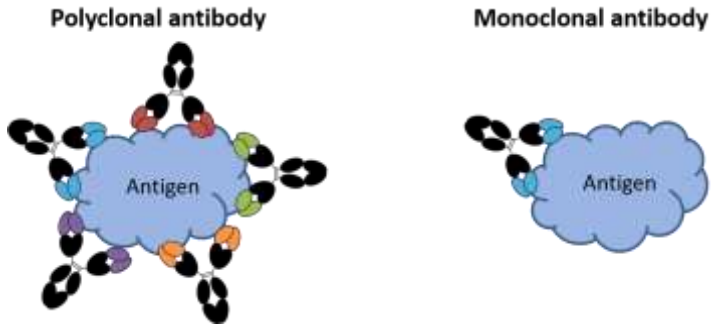


Figure 62 Schematic illustrations of polyclonal and monoclonal antibodies binding with antigens (Pavlou & Belsey, 2005).

The mono specificity provided by MAbs is useful in evaluating changes in molecular conformation, and in identifying single members of protein families, for example the insulin family. However, the monospecificity of MAbs may also limit their usefulness. For instance, small changes in the structure of an epitope, perhaps as a consequence of denaturation, can markedly affect the function of MAb. By contrast, as PAbs are heterogeneous and recognise a host of antigenic epitopes, the effect of change on a single or small number of epitopes is less likely to be significant. PAbs are also more stable over a broad pH and salt concentration, whereas MAbs can be highly susceptible to small changes in both (Pavlou & Belsey, 2005).

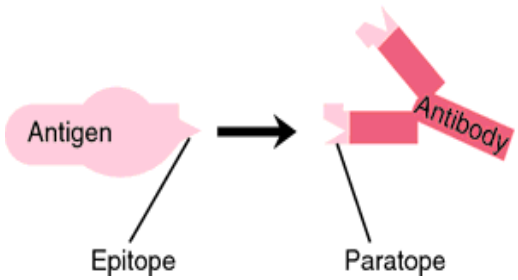


Figure 63 Antigens contain antigenic determinants (epitopes) and antibodies contain antibody combining sites paratope (Copstead & Banasik, 2015).

An epitope is a piece of a particle to which an immune response ties. Epitopes can be made out of sugars, lipids or amino acids, though as a rule, they are built of amino acids. Epitope labels are added to an atom as a rule protein, which an examiner must envision. Visualisation can happen in a gel, a western blot or naming through immunofluorescence. In total, an epitope is approximately five or six amino acids long; accordingly, a regular full-length protein contains a wide range of epitopes against which antibodies can tie. Also, for any given protein arrangement, one will typically find that different antibodies will perceive the protein. Each of these antibodies ties to a particular epitope situated on that protein (Smith-Gill, 1994).

Binding between the antibody and the epitope occurs at the Antigen Binding Site. One can find continuous epitopes, which are linear sequences of amino acids and discontinuous epitopes that exist only when the protein is folded into a particular conformation (Smith-Gill, 1994).

5.3.1.2 Immunoprecipitation

Immunoprecipitation is one of the most widely used methods for antigen investigation and isolation (Harr & Johnson, 2015). The principle of IP is a direct assay, by using a specific antibody against the target antigens to react together, such as a protein sample (insulin). The binding side of the antibody with antigens will tag it to the beads (Protein A) and then precipitate Finally, the unbound antigen and antibody will be washed away by denaturing buffers, and antigens will be eluted and analysed using SDS-PAGE, followed with immune blotting to confirm the identity of the antigen **(**Harr & Johnson, 2015).

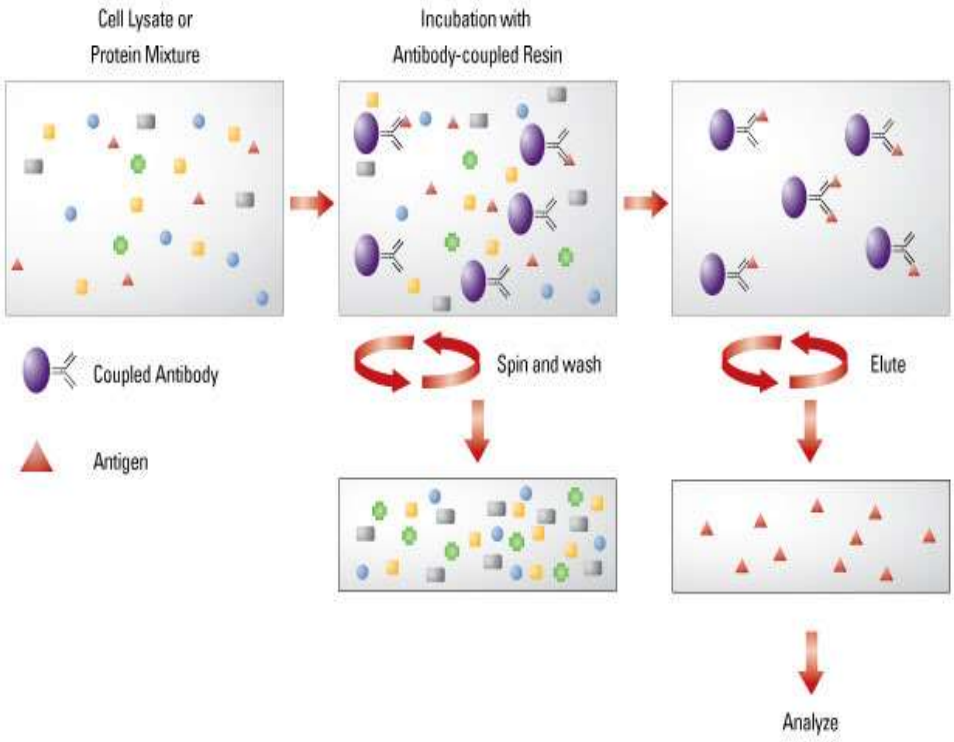


Figure 64 Schematic diagram to illustrate the immunoprecipitation principle, obtained from www.lifetechnologies.com.

5.3.1.4. Chemiluminescence system

Chemiluminescence is the western blot and ELISA detection method of choice in most protein laboratories, as it provides the greatest sensitivity and convenience for detection with film or digital imaging equipment. Chemiluminescense substrates for horseradish peroxidase (HRP) are two-component systems consisting of a stable peroxide solution and enhanced luminol solution. To make a working solution, the equal volumes of the

two components are mixed together. When incubated with a blot or micro wells plate on which HRP-conjugated antibodies (or other probes) are bound, a chemical reaction produces light that can be detected by film in western blot, or a sensitive camera. ELISA is measured in relative luminescence units (RLU), using a microplate reader with chemiluminescence capability (luminometer).

5.3.2 Test to measure biological activity of insulin

Glucose-6 phosphate is a key sugar intermediate that allows glucose to enter cells and subsequently either metabolic pathways or storage. Glucose-6 phosphate can enter the glycolytic pathway or the pentose phosphate shunt, or it can be stored as glycogen or starch. The biological activity of insulin can be measured by quantifying the Glucose-6 phosphate (G6P) concentration in cells after glucose is phosphorylated by hexokinase due to stimulation by insulin. G6P is a monophosphorylated derivative of glucose with the phosphate group attached to C-6. Moreover, insulin is required to convert glucose to G6P (Champe, Harvey & Ferrier, 2005).

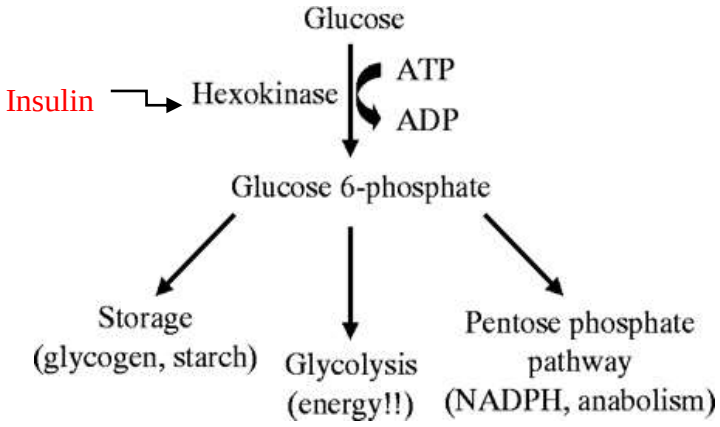


Figure 65 Glucose phosphorylation pathways (Wilson, 2003).

Sigma's glucose-6 phosphate assay kits are a simple, sensitive and rapid means of quantifying glucose-6 phosphate in a variety of samples. G6P is generated when glucose is phosphorylated by hexokinase or glucokinase, or by the conversion of glucose-1-phosphate by phosphoglucomutase during glycogenolysis. G6P lies at the beginning of both glycolysis and the pentose phosphate pathways, and is determined by an enzyme assay, which results in a colorimetric (450 nm) product, proportional to the G6P present. Typical sensitivities of detection are between 1-30 nM

The effects of insulin at the cellular level are initiated by the binding of insulin to its plasma membrane receptor (Figure 65). The ligand-induced autophosphorylation of the receptor is followed by the phosphorylation of endogenous substrates to mediate the transmission of an insulin-signaling pathway (Gardner, 1993; Stumpo, 1984).

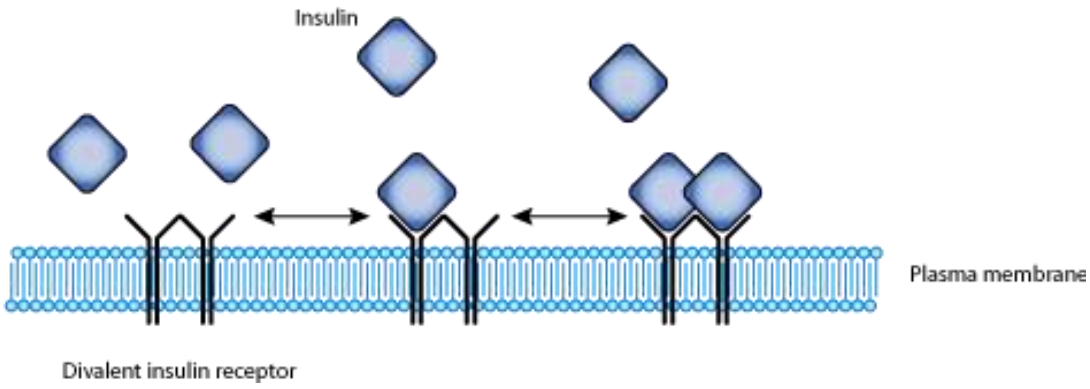


Figure 66 Schematic diagram illustrating insulin binding to cellular receptors to stimulate uptake of glucose in the blood stream (Wantan & Quon, 2000).

Several hepatic cell lines are recommended for use in assays of insulin activity: BALB/c 3T3, HepG2, NIH3T3 and Bel7402.BALB/c3T3 (Zhang et al., 2008). HepG2-C3A was used in this study; it was isolated from a liver biopsy of a Caucasian male aged 15 years, with a well differentiated hepatocellular carcinoma as a clonal derivative of HepG2A, which is considered a highly analytical in vitro model for drug metabolism studies (Lyer et al.,2010).

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5.4. Materials and Methods

5.4.1. Materials

5.4.1.1 General materials

Raw camel milk was obtained from Bradford, UK (Kamelus, the home of 'White Gold'). Camel milk from this source was subsequently discontinued. The decision was made to obtain pasteurised skimmed camel milk from Saudi Arabia (Al-turath alsaudi) to continue with the experiments.

5.4.1.2 Enzyme-linked immunosorbent assay (ELISA) anti-insulin antibodies

All anti-insulin antibodies were obtained from Mercodia, as a full kit.

Mercodia mouse anti -human insulin antibody (cat no: 10-1113-01). Mercodia mouse anti-human iso-insulin antibody (cat no: 10-1128-01). The difference between iso insulin and human insulin antibodies is that the iso insulin antibody is characterised by higher crosslinking with insulin analogues than human insulin (Woitiski et al., 2010). Mouse anti bovine -insulin antibody (cat no: 10-1201-01).

Materials in the Mercodia kit:

Coated plate with mouse monoclonal anti-insulin antibodies. Calibrators (recombinant human insulin) 1, 2, 3, 4, 5 5 vials 1000 µL ready for use, colour coded yellow. Detection antibody: Mouse monoclonal anti-insulin antibodies. HRP linked antibody: Goat anti mouse IgG. Enzyme Conjugate: Peroxidase conjugated mouse anti human -insulin

5.4.1.3 Western blot insulin antibodies

Polyclonal rabbit anti human insulin antibody was obtained from NOVUS (cat. no.: NBP 1-45662). Monoclonal mouse anti Human insulin/proinsulin antibody was obtained from Thermo Scientific (cat. no.: MA1-83256. Rabbit Anti-mouse IgG (secondary antibody) was obtained from Sigma (cat. no.: A9044). Goat Anti-rabbit IgG (secondary antibody) was obtained from Sigma (cat. no.: A3687). Insulin recombinant protein (control sample) was obtained from Thermo Scientific (cat. no.: RP-10908).

5.4.1.4 Western blot and polyacrylamide gel electrophoresis (PAGE)

Phosphate-buffered saline (PBS) was obtained from Sigma (cat. no.: P4417).

BCIP®/NBT Liquid Substrate System, ready to use solution (B1911).

Insulin, human recombinant, zinc solution (Gibco®) was obtained from Life Technologies (cat. no.: 12585-014). iBlot® Transfer Stack, PVDF, Mini was obtained

from Life Technologies (cat. no: IB4010-02). NuPAGE® Novex® 12% Bis-Tris Protein Gels were obtained from Life Technologies (cat. no.: NP0341PK2). SeeBlue® Prestained marker was obtained from Life Technologies (cat. no: LC5625). ECL™ Western Blotting Detection Reagents were obtained from Sigma (cat. no.: GERPN2209). XCell SureLock™ Mini-Cell was obtained from Invitrogen (cat. no.: EI0001). Coomassie stain was obtained from Bio-rad (cat. no.: 161-0786).

5.4.1.5 Immunoprecipitation

Protein A-Sepharose *Staphylococcus aureus* obtained from Sigma (cat. no.: P3391).
Molecular weight: 42KDa
No. of binding sites for IgG: 4
Binding capacity: 250 µg
Optimum pH: 7.4

5.4.1.6 Tissue culture assay to measure insulin activity

Glucose-6 phosphate assay kits were obtained from Sigma (cat. no.: MAK014) and Abcam (cat. no: ab83426).
HepG2 and HepG2-C3A cell lines (derived from human hepatocellular carcinoma) were obtained from Nanosafety Group (School of Life Sciences, Heriot-Watt University).
Black, solid-bottom micro plates, 96-well assay.
Fetal bovine serum was obtained from Sigma (cat. no.: F2442).
Eagle's Minimum Essential Medium was obtained from Sigma (cat. no.: M 2279).
Trypsin-EDTA solution was obtained from Sigma (cat. no.: T4049).
Corning® cell culture flasks with a surface area of 25 cm^2, angled neck and cap (phenolic-style) were obtained from Sigma (cat. no.:CLS430372).
Pluronic F68 was obtained from Sigma (cat. no: P1300).
Bactopeptone was obtained from Fisher Scientific (cat. no.: 50-254-269).
L-glutamine was obtained from Sigma (cat. no: G3126).

5.4.2 Methods

5.4.2.1 Tests to determine physical presence of insulin

5.4.2.2.1 ELISA (enzyme linked immunosorbent assay).

1. Prepare enzyme conjugate 1X solution and wash buffer with 1X solution.
2. Prepare sufficient microplate wells to accommodate calibrators, controls and samples with duplicates.

3. Pipette 25 µlL each of calibrators, controls and samples into appropriate wells.
4. Add 100 µL of enzyme conjugate 1X solution to each well.
5. Incubate on a plate shaker (700-900 rpm) for 1 hour at room temperature (18–25°C).
6. Wash six times with 700 µL wash buffer 1X solution per well. Discard the reaction volume by inverting the microplate over a sink. Add 350 µL wash buffer 1X solution to each well. Discard the wash solution, tap firmly several times against absorbent paper to remove excess liquid. Repeat five times. Avoid prolonged soaking during washing.
7. Add 200 µL Substrate TMB into each well.
8. Incubate on the bench for 15 minutes at room temperature (18–25°C).
9. Add 50 µL Stop Solution to each well. Place plate on a shaker for approximately 5 seconds to ensure mixing.
10. Read optical density at 450 nm and calculate the results; read within 30 minutes.
Three different types of ELISA were performed using different anti insulin antibodies. The assay was performed using raw, pasteurised skimmed camel milk, casein and whey. Bovine serum albumin was used as the negative control protein, as well as the calibration sample provided by the supplier, which contained no insulin.

5.4.2.2.2 Immune precipitation

Protein A-sepharose from *S. aureus* was used to precipitate the insulin or insulin-like protein from pasteurised camel milk. The beads contain molecules that bind non-specifically to the Fc region of IgG of many species (Page & Thorpe, 2002). The antibody interacted with the target antigen, insulin in the milk. Unbound protein has washed away with PBS and the bound insulin was eluted with SDS buffer

Preparation of the beads

Beads come as a powder, 100 mg was incubated in 1 ml 0.1 M PBSfor 1 hr so they swell up, then centrifuged 1000 rpm for 5 minutes, supernatant was remove and discard.

Immuneprecipitation

The (1 ml) liquid milk sample was incubated with 5µg antibody and 5 mg beads for 1 hour at 4°C, under gentle agitation. After that, 1 ml PBS containing 0.1% BSA were added, and mixed for 1 hr, followed by rinsing in PBS 2 X. and removal of supernatant.

Elution process and buffer

Elute 5 mg of beads by heating in 50 µl of 2X SDS for 10 min at 50°C.

The proteins in the elution buffer were run on SDS-PAGE gel under reducing (addition of 2-mercaptoethanol) and non-reducing conditions.

5.4.2.2.3 SDS PAGE & immune blot

SDS- PAGE has described in Chapter 3 Section 3.2.2.8

This was followed by the western blotting method, as described by Life Technologies **(**2013). The protein bands on the SDS-PAGE gel were transferred onto the PVDF membrane via the IBlot® Transfer Stack (semi-dry method). The membrane was wet with ethanol or methanol to reactivate; the avoidance of this step would have caused the transfer of the bands from the gel to the PVDF membrane to be unsuccessful. Following the transfer, the PVDF membrane was stained with Ponceau S colour to ensure that all bands were properly transferred from the gel to the membrane. In addition, the gel was stained with Coomassie blue stain to determine the amount of protein left in the gel. The PVDF membrane was incubated overnight with mouse anti human insulin antibody at 4°C, before being thoroughly washed, three times, with phosphate buffer saline (one tablet dissolved in 200 mL of deionised water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25°C) (100 ml) for five minutes each time. Then, HRP labelled rabbit anti mouse IgG secondary antibodies were prepared according to the manufacturer's instructions. The membrane was then incubated with the secondary antibody for 1 hour at 37°C. The membrane was then washed again with phosphate buffer, as described above. Alkaline phosphatase enzyme was used to visualised bands by incubated the membrane with ready to use Alkaline phosphates ready to used reagent in 37ºC for 3 minutes, until purple bands started to develop and appear on the membrane .

Chemiluminescent detection

Luminol Enhancer Solution Substrate (LES) working solution was prepared by mixing equal parts of the Peroxide Solution and the (LES). Membrane was incubator with LES for 1 minute at room temperature, then the membrane was removed from solution, and placed in a plastic sheet protector and placed in Bio rad Chemiluminescent detection system. The reaction occurs in the present of HRP (horseradish peroxidase) that catalyzes luminol oxidation in the presence of hydrogen peroxide. Kodak Bio Max light film was used for X ray film exposure. The film was developed in an automated processor, and the signal was qualitied by scanning with molecular imager.

Measurements were done with a microplate reader (Spectra MAX M5). Dot-blot tests were performed using different concentrations of insulin to determine the minimum antibody capacity for detection. The same approach was repeated to visualise the interaction between the primary and secondary antibodies.

5.4.2.3 Tests to measure biological activity of insulin

The glucose 6 phosphates kit included:

- Glucose-6-Phosphate Assay Buffer
- Glucose-6-Phosphate Probe
- Glucose-6-Phosphate Enzyme Mix
- Glucose-6-Phosphate Standard

Preparation of G6P Standards

In this stage, 10 mL of the 100 mM G6P standard solution was diluted with 990 ml of water to prepare a 1 mM standard solution, and 10 mL of the 1 mM standard solution was added to 990 mL of water to make a 0.01 mM G6P standard solution.

Then, 0, 1, 2, 3, 4, and 5 µL (for 10–50 pmole range) of the 0.01 mM G6P standard solution was added to a 96 well plate, generating 0, 10, 20, 30, 40 and 50 µl/well standards or 0 (blank), 100, 200, 300, 400, and 500 picomole/well standards. Glucose-6-Phosphate Assay Buffer was added to each well to bring the volume to 50 picomole/ µl. The plate was covered with aluminium foil and left in the incubator at 37^{o} C for 5 min.

Then, the following was mixed: 100µl of mixed G6P enzyme + 100µl of Glucose-6-Phosphate Probe + 100µl of substrate + 2200µl of the buffer. The enzymes reagent was pipetted in 44µl amounts into each well quickly, then the plate was measured at a fluorescence intensity of λex = 535 nm/λem = 587 nm.

Preparation of hepatic cells

The main objective of this assay was to measure insulin activity using the camel milk fractions with human insulin serving as the positive control. HepG2- C3A hepatic cell line was used; these cells have insulin receptors and have previously been used for measuring insulin activity (Iyer et al., 2010). Cells were incubated in a medium containing low-glucose (1mg/ml) medium 0.05% pluronic F68, 0.2% bactopeptone, and 2 mM glutamine, 2 mM glutamine, 10 kU/l penicillin and 100 mg/l streptomycin. Cells were allowed to continually divide in the flask surfaces. The cells were fed every three days with the medium to ensure healthy cell growth, as recommended by the manufacturer. The cells were counted to obtain a concentration of 4.4×10^5 CFU. Two

plates were seeded with cell lines in the sixth phase of growth. Human Insulin was used as a positive control at two different insulin concentrations (145 and 250 ng/µl). The camel milk sample protein concentration was 500 µg/µl. Cells were incubated at 37°C overnight, and each sample was measured at two time points at 24 hours and 48 hours, Supernatant was collected and stored at -80°C to measure the glucose-6-phosphate at a later point. Cells were counted to obtain a concentration of $4.4 \times 10^{5.}$ A CFU (colony-forming unit) and was rapidly homogenised in 2–3 volumes of ice cold PBS. The samples were centrifuged at 13,000 g for 10 minutes to remove insoluble material; they were then deproteinised with a 10 kDa MWCO spin filter and then added to the microtitre plate, where the reaction took place. Samples were brought to a final volume of 50 µL/well with Glucose-6-Phosphate assay buffer and transferred into duplicate wells of the 96 well plates.

Assay Reaction

44µl/well of the appropriate Reaction Mix was added to each reaction well. Microplate wells were mixed using a horizontal shaker, or by pipetting. The reaction was incubated for 5 minutes at 37°C, all the while being protected from light. Finally, the fluorescence intensity was measured (lex = 535/ lem = 587 nm) reading was done using microplate reader (Spectra MAX M5)

Calculating the background for the assays

The background can be corrected for by subtracting the blank value from all readings; background values can be significant and so must be subtracted from all readings. The values obtained from the appropriate Glucose-6-Phosphate standards were used to plot a standard curve. The blank sample value was subtracted from the sample reading to obtain the corrected fluorescence measurement. Using the corrected fluorescence measurement, the amount of Glucose-6-Phosphate present in the sample was determined from the standard curve. Concentrations of Glucose-6-Phosphate were been calculated as follows:

- Sa/Sv = C
- Ay = Amount of Glucose-6-Phosphate in unknown sample (nmole) from standard curve
- Sv = Sample volume (mL) added into the wells.
- C = Concentration of Glucose-6-Phosphate in sample Glucose-6-Phosphate molecular weight: 260.14 g/mole sample calculation amount of Fructose-6-Phosphate

- (Sa) = 20 pmole assay volume
- (Sv) = 50 mL Concentration of Fructose-6-Phosphate in sample 20 pmole/50 mL = 0.4 nmole/mL 0.4 pmole/mL ´ 260.14 pg/pmole= 104.0 pg/m

5.5 Results and Discussion

5.5.1 ELISA results

5.5.1.1 Insulin determination in pasteurised camel milk using human iso-insulin antibody

Mouse anti Human iso-insulin antibody was used to measure the total insulin in a sample of raw and pasteurised camel milk. Iso-insulin has a high cross-reactivity to insulin analogues, and measures both the endogenous insulin and possible analogues (Whitmore et al., 2012).

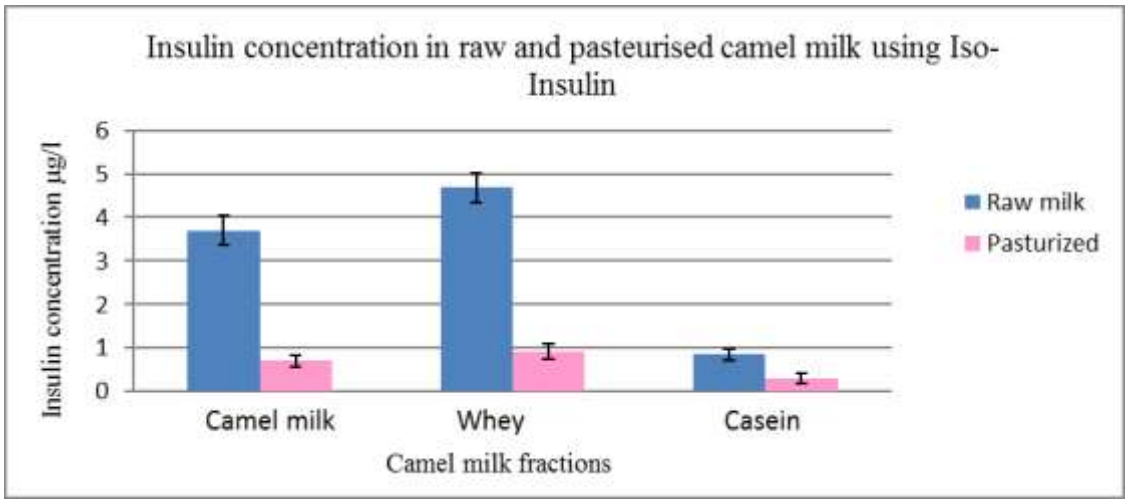

Figure 67 The different insulin contents in pasteurised and raw camel milk, using iso insulin antibody.

Table 24 Protein and insulin concentration in raw and pasteurised camel milk; insulin content was measured with mouse anti human iso-insulin antibody; ELISA.

Protein	Raw camel milk (µg/l)	Pasteurised camel milk (µg/l)
Camel milk	3.7 ± 0.47	0.7± 0.67
Camel whey	4.69 ± 0.45	0.91 ± 0.83
Camel casein	0.85 ± 0.85	0.3 ± 0.39
BSA	0.01 ± 0.12	0.01 ± 0.05

The results shown in Figure 67 and Table 24 reveal that the insulin concentration is less in pasteurised milk compared with in raw milk. This confirms the results of Ley et al. (2011), who report that pasteurisation reduces insulin concentrations in donor human milk pasteurised at 62.5 ºC for 30 minutes. Ollikainen and Muuronen (2013) also report a decrease an insulin concentration of heat-treated bovine milk (Figure 68) using the ELISA method and anti-human insulin antibody.

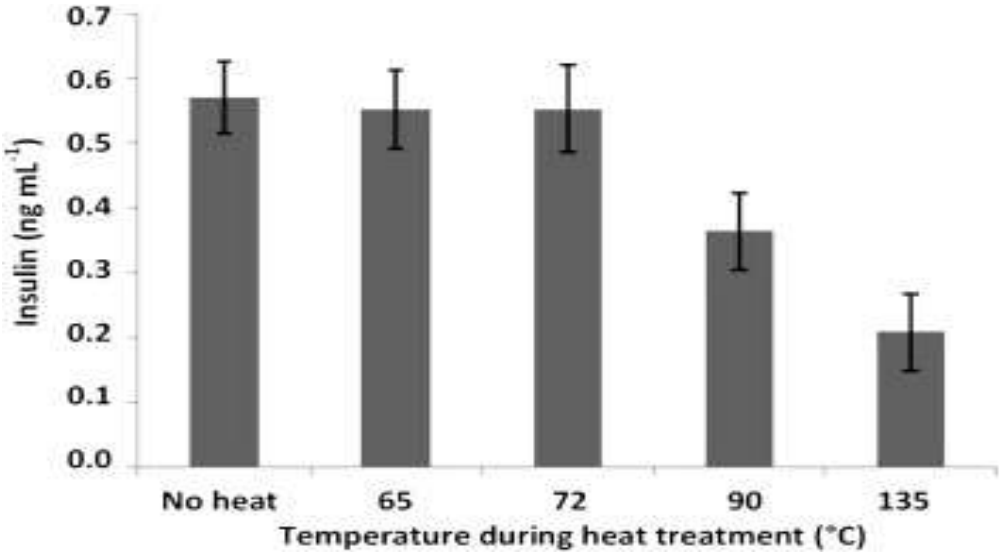

Figure 68 The insulin concentration in bovine milk heated at different temperatures compared to an unheated sample (Ollikainen & Muuronen, 2013).

Table 25 and Figure 69 show that the reaction between insulin antibody and insulin-like protein in camel milk fractions is significantly higher for raw camel milk compared with pasteurised milk.

5.5.1.2 Insulin content in camel milk fractions using different insulin antibodies

The purpose of this step was to investigate the difference in antibody reactions in camel milk fractions.

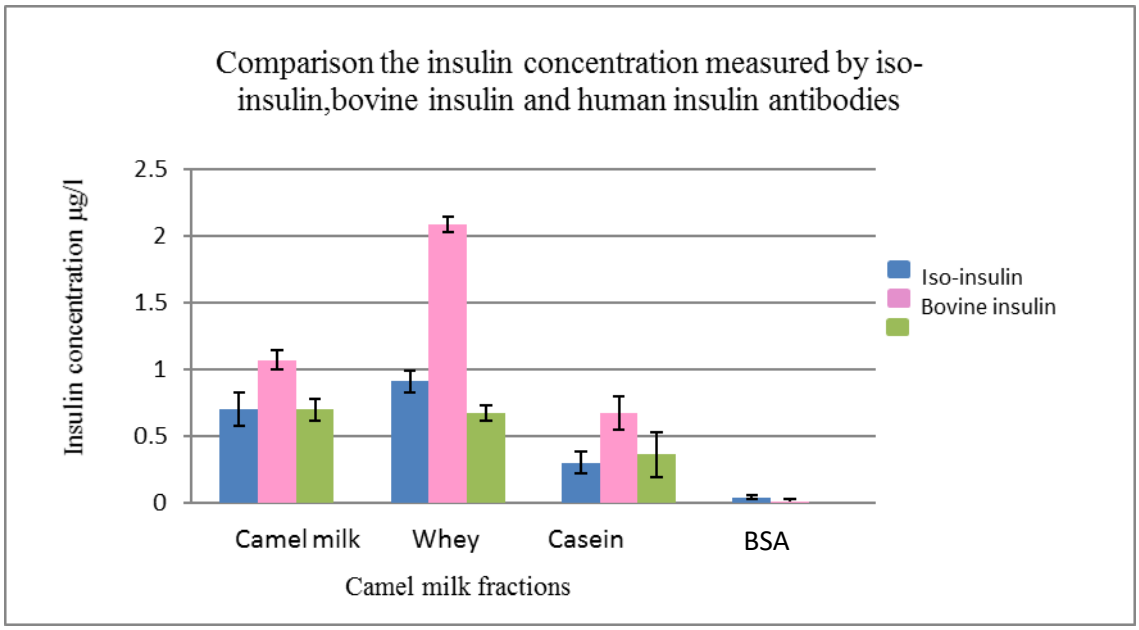

Figure 69 Insulin content in pasteurised camel milk by iso-insulin, human insulin and bovine anti-insulin ELSIA kits.

Table 25 Insulin content using different insulin antibodies

Protein	Isoinsulin antibody	Bovine insulin antibody	Human insulin antibody
Camel milk	0.7± 0.67	1.07± 0.92	0.7 ± 0.70
Camel whey	0.91± 0.83	2.09 ±0.20	0.67± 0.43
Camel casein	0.3± 0.39	0.67± 0.55	0.36± 0.75
BSA	0.01± 0.5	0.01± 0.06	0.02± 0.24

All ELISA kits showed that most antigens against anti-insulin antibodies were present in the whey fraction of camel milk. Table 25 shows the insulin concentration of pasteurised camel milk fractions detected using three different anti-insulin antibodies. The highest concentration was found with anti-bovine insulin antibody, compared with iso-insulin and human anti-insulin, with concentrations of 1.07, 0.7 and 0.7 µg/l respectively.

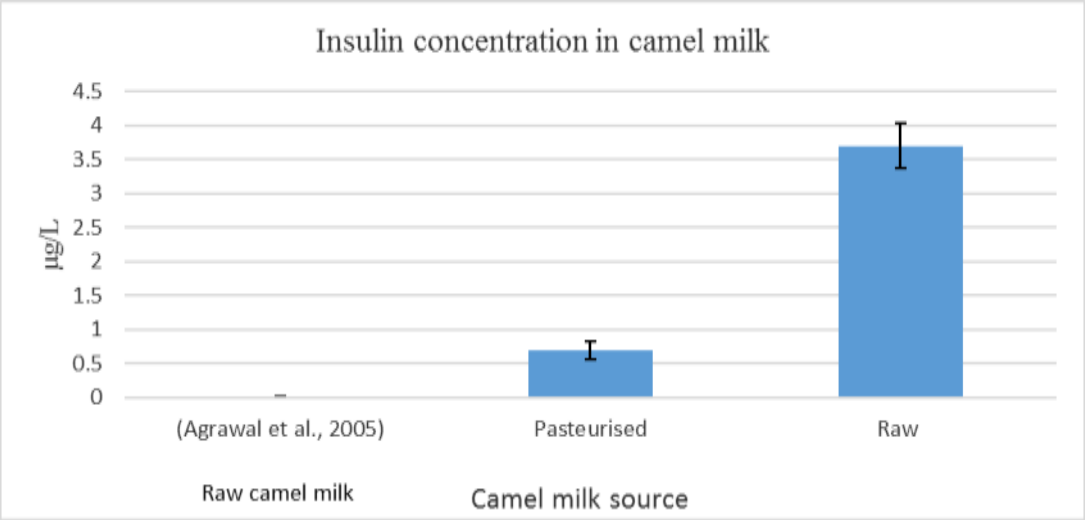

Figure 70 Insulin concentrations in pasteurised and raw milk camel milk compared with the results of Agrawal et al. (2005).

Figure 70 shows a significant difference in camel insulin measured with anti-insulin anti body comparing with what has found by Agrawal (2005) using the radio immune assay and the insulin concentrations in the project, using the ELISA test. The ELISA showed high interactions with the three different anti insulin antibodies. Studying the insulin activity in milk was an important step here for determining whether insulin or insulin-like proteins are present in camel milk.

5.5.2 Western blot and SDS-PAGE results

5.5.2.1 Detection of the positive control

Human insulin was included in the western blot as a positive control. Several experiments were performed to detect the affinity of anti-insulin antibody with the insulin sample (positive sample). This was a very important step in confirming the validity of the antibody and insulin itself before applying it to the milk sample.

Figure 71 Western blot of control sample (human insulin). Monoclonal anti-bovine insulin antibody was used as the primary antibody. The secondary antibody was rabbit anti human IgG labelled with alkaline phosphates.

Western blotting was repeated several times to detect the positive control insulin, without any result. Incubation for one or two hours at 37°C is recommended by most manufacturers for anti-insulin antibody detection; the insulin band was finally detected when the antibody was incubated overnight with insulin at 4ºC, as recommended by Lieu et al. (2014). The band was clear and sharp at 5.8 kDa in size, shown in Figure 71, which is consistent with the molecular weight reported for insulin (Athanasios et al., 2005).

5.5.2.2 Insulin detection in milk fractions by western blot

Anti-human insulin antibody was used for insulin detection in camel milk. Polyclonal anti-human insulin was used, as it can detect any insulin family by recognising multiple epitopes on the same antigen.

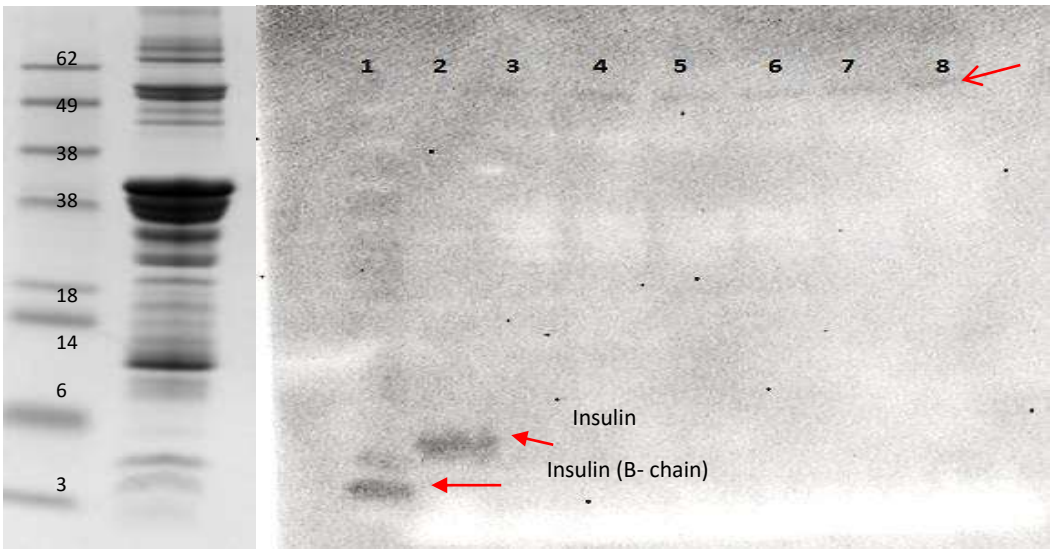

Figure 72 Left image: Left image: SDS PAGE of molecular weight markers (first lane) and camel milk (second lane). Right Image: western blot of pasteurised camel milk using Polyclonal rabbit anti human insulin antibody. The labelled secondary antibody was HRP Goat Anti-rabbit IgG, which visualised using the chemiluminescence system.

The results of the blot illustrated in Figure 72 show that the insulin antibodies do not detect a band corresponding to insulin molecular weight in camel milk. The only bands that appear with camel milk samples are the high molecular band (approximately 62 kDa). Bano et al. (1994) noted the size of protein present in bovine milk and identify it as mammary-derived growth factor 1 (MDGF1), while Playford et al. (2000) consider it to be a new protein that belongs to the category of growth factors. The functions of this protein has not been fully identified yet; its only known function is that it stimulates the growth of mammary cells and enhances collagen production (Bano et al., 1992).

Another explanation could be that, in bovine serum, there are insulin-like growth factor (IGF) molecules that belong to the insulin family. McGuire et al. (1992) report three different IGF-binding protein fractions with apparent molecular weights of > 200, 140 to 160, and 45 to 64 kDa. No previous information has been reported about this protein yet (McGuire et al., 1992).

5.5.2.3 Detection of insulin in reduced and non-reduced camel milk samples.

In order to investigate the possibility of the 62 KDa band consisting of disulfide bonds in insulin polymers, a sample was treated with the reducing agent 2-Mercaptoethanol. A sample was reduced by inclusion of 2-Mercaptoethanol in the sample buffer before SDS

PAGE. 2-Mercaptoethanol or β-mercaptoethanol are often used to reduce disulfide bonds in proteins for the purpose of analysis.

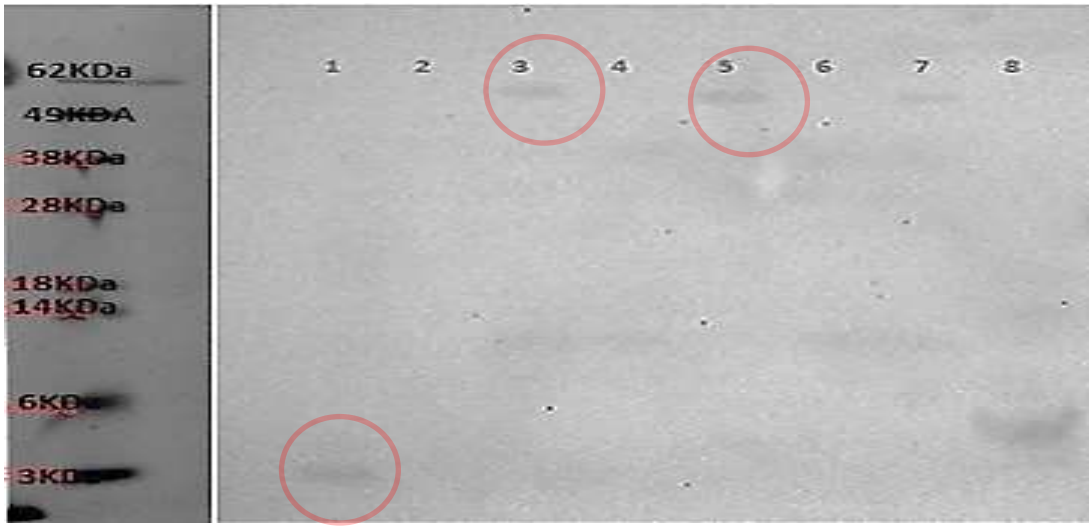

Figure 73 Western blot of pasteurised camel milk. Left: stained molecular weight markers. Right: Lane 1, human insulin; Lane 2, reduced pasteurised camel milk; Lane 3, non-reduced camel milk; Lane 4, reduced whey protein from camel milk; Lane 5, non-reduced whey from camel milk; Lane 6, reduced casein from camel milk; Lane 7, non-reduced casein from camel milk; and Lane 8, non-reduced insulin.

Polyclonal rabbit anti human insulin antibody was used as the primary antibody. And secondary antibody was Goat Anti-rabbit IgG labelled with HRP.

The western blot analysis in Figure 73 shows that the antibody reacted with 62 kD in the non-reduced sample (lanes, 3, 5 and 7) but did not react with the reduced samples (lanes 2, 4 and 6). This result shows that broken disulfide bonds lead to change in protein conformation, and then destroy the linear epitopes. Another explanation might be that the 62 KDa band consist of polymers with a low molecular weight protein bound by disulphide bonds. For example, an insulin molecule could be dissociated into small peptides < 2.5 KDa, which would lead it to not to be detected by antibodies. Human insulin 5.8 KDa contains two inter-chain disulfide bonds between the A and B chains (Chang et al., 2003), which result in < 3KDa for the B chain and 2.5KDa for the A chain (Hellman et al., 1973). The insulin control in lane 1 of **Error! Reference source not found.** (circled in red) is the β chain of human insulin. The high molecular weight bands of lanes, 3, 5 and 7 could contain insulin-like proteins that react with antibodies.

5.5.2.4 Insulin detection in milk fractions using immune precipitation (protein A)

An immonoprecipitation experiment was conducted to concentrate insulin and insulin-like proteins where they were too dilute in camel milk.

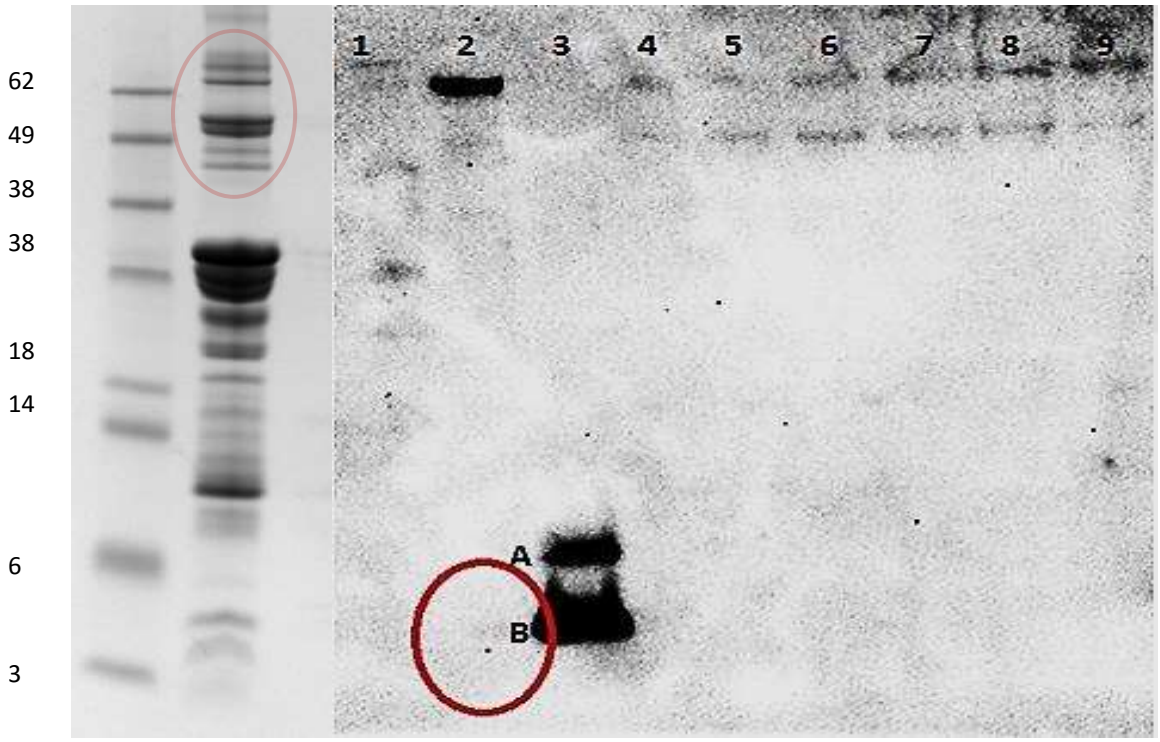

Figure 74 Left figure: SDS PAGE gel molecular weight markers of camel milk

Right: SDS PAGE gel molecular weight markers of camel milk. Right Figure: Lane 2 immune precipitated camel milk sample with protein A beads using monoclonal mouse anti Human insulin/proinsulin antibody. Lane 2: monoclonal mouse anti Human insulin/proinsulin antibody

Lane 3 shows a reaction in two bands with the positive control insulin, marked as A and B. The antibody is bound to the human insulin (B) and pro-insulin (A), the pro-insulin band that is bigger in size than insulin itself (Cohen et al., 1986). Assuming that the insulin was too diluted in milk, pasteurised camel milk serum was precipitated with protein A, and the sample was loaded in Lane 2, resulting in a band at ~62 KDa, showing a significantly high affinity to antibodies.

All western blots of camel milk and fractions precipitated with protein A show very strong bands at 62 KDa. However, this band does not correspond to the reported size of the camel hepatic insulin (Baragob et al., 2011), which is 5.8 KDa. No previous studies have examined immunoprecipitated samples of camel milk and anti-insulin antibody to

compare the results. Furthermore, the proteins in camel milk have not yet been fully identified, particularly those corresponding to the band of size ~62 KDa.

5.5.2.5 Western blot with different camel milk fractions (whey, casein and whole camel milk)

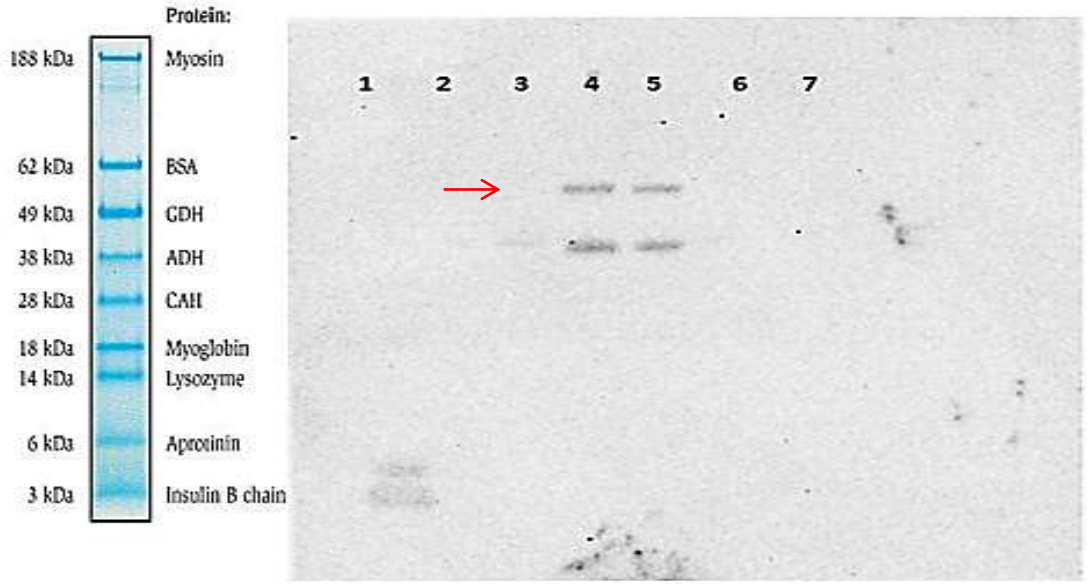

Figure 75 Western blot of raw camel milk protein transferred from 12% protein gel to the PVDF membrane. Polyclonal rabbit anti human insulin antibody was applied, and bands were visualised by chemiluminescent system using HRP Goat Anti-rabbit IgG**.**

Figure 66 bands appeared at sizes of ~62 and ~49 kDa for the whey protein fraction of raw camel milk and faint bands for 300 µg camel milk (Lane 3). No immune reaction was evident in the casein fraction; however, the band size ~62 kDa was stable in pasteurised milk. Neither band has yet been defined in previous studies. In conclusion, no band was found in camel milk of low molecular weight (5.8kD) similar to human insulin. Elamin et al. (2014), note that the size of camel insulin in camel blood is 5,800 Daltons, which is similar to the known figures for other species. The western blot experiments did not show any interaction with the insulin antibody at this size. However, polyclonal and monoclonal antibodies detected high molecule sizes of~ 62 and~ 49 KDa. This could be an insulin-like protein that has not yet been identified in camel milk.

5.5.3 Insulin biological activity measuring by *in vitro* study

The main objective of this assay was to measure insulin biological activity using camel milk, using human insulin as the positive control. Camel milk has previously been shown to have anti-diabetic factors by *in vivo* studies, but currently not enough studies have been performed *in vitro.*

5.5.3.1 Insulin biological activity (control samples) in two different concentrations and two different incubation times

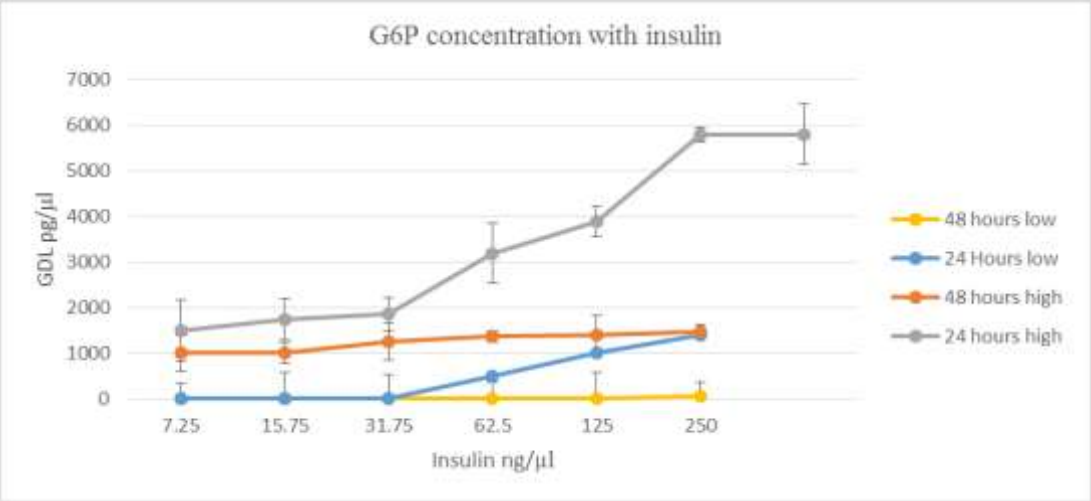

Figure 76 Concentration of G6P at 24 and 48 hours for two different concentrations of insulin

Insulin has applied at concentration of (149 and 250 ng/µl). Different concentrations according to the suggestion of Zhang et al. (2008) .The results show reasonable G6P concentrations in the first 24 hours. Nevertheless, after 48 hours incubation time, the insulin showed no biological activity. These results remain unexplained.

5.5.3.2 Camel milk insulin biological activity result compared with insulin after 48 hours at different incubation times

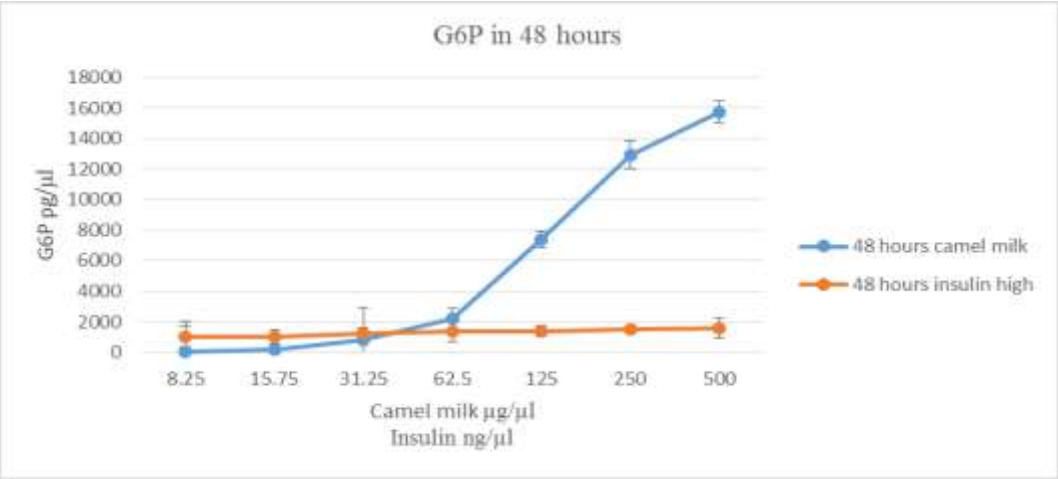

Figure 77 Concentration of G6P, with camel milk, compared to insulin after 48 hrs.

Figure 77 shows a significantly high concentration of G6P after incubation with camel milk with cell lines for 48 hours. Whereas, insulin as a positive control show very low G6P concentration after 48 hours compared to camel milk, which showed activity starting at concentrations of 31 µg/µl, and later improved.

The purpose of the next graph was to compare the insulin activity of camel milk samples at different incubation times (24 and 48 hours).

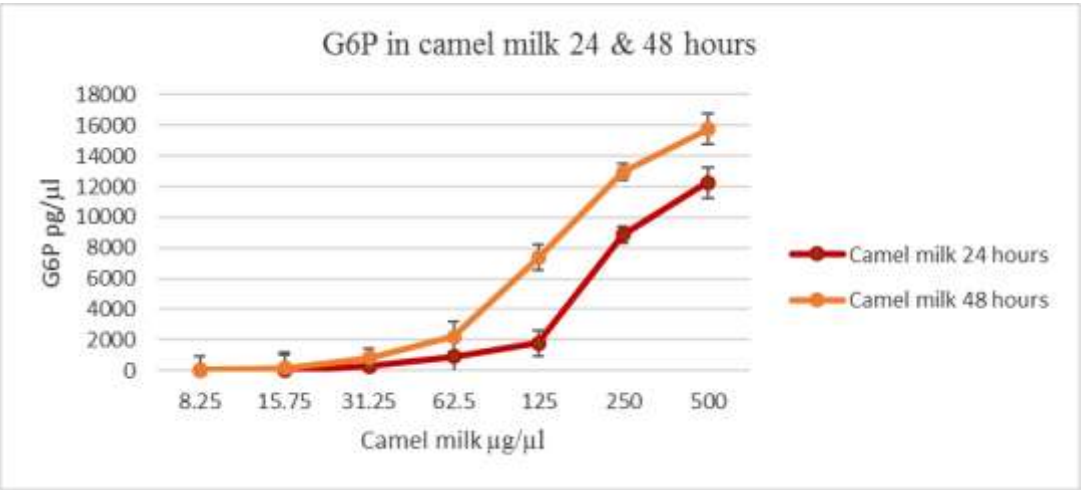

Figure 78 Concentration of G6P, with camel milk sample in different incubation times, 24 and 48 hours.

Figure 78 shows that camel milk incubated for 48 hours resulted in significantly high insulin activity, compared with the camel milk sample incubated for 24 hours. These results confirm that insulin biological activity is present in camel milk. The activity of the insulin in camel milk begins at a minimum concentration of camel milk protein of 31µg/ul. The activity of insulin-like protein increases as milk protein increases

The G6P activity profiles reports here for camel milk could be explained by the fact that camel milk may contain insulin or insulin-like proteins. Some recent studies have suggested that insulin signalling plays a role in modulating the activities of excitatory and inhibitory receptors, such as glutamate and γ-aminobutyric acid GABA receptors (Zhao et al., 2004). Limon et al. (2014) report that orally administered γ-aminobutyric acid GABA has several beneficial effects on health, including the regulation of hyperglycaemic states in humans. These effects are similar to those reported for camel milk, which has been found to have significantly more bioavailable GABA compared with bovine and human milk, and is able to activate GABA receptors (Limon et al., 2014). Pancreatic islet β-cells produce large amounts of γ-aminobutyric acid (GABA) in non-diabetics, which is co-released with insulin. It has also been reported that β-cells express GABA receptors, the activation of which increases insulin release (Bansal et al., 2011).

5.6 Conclusions

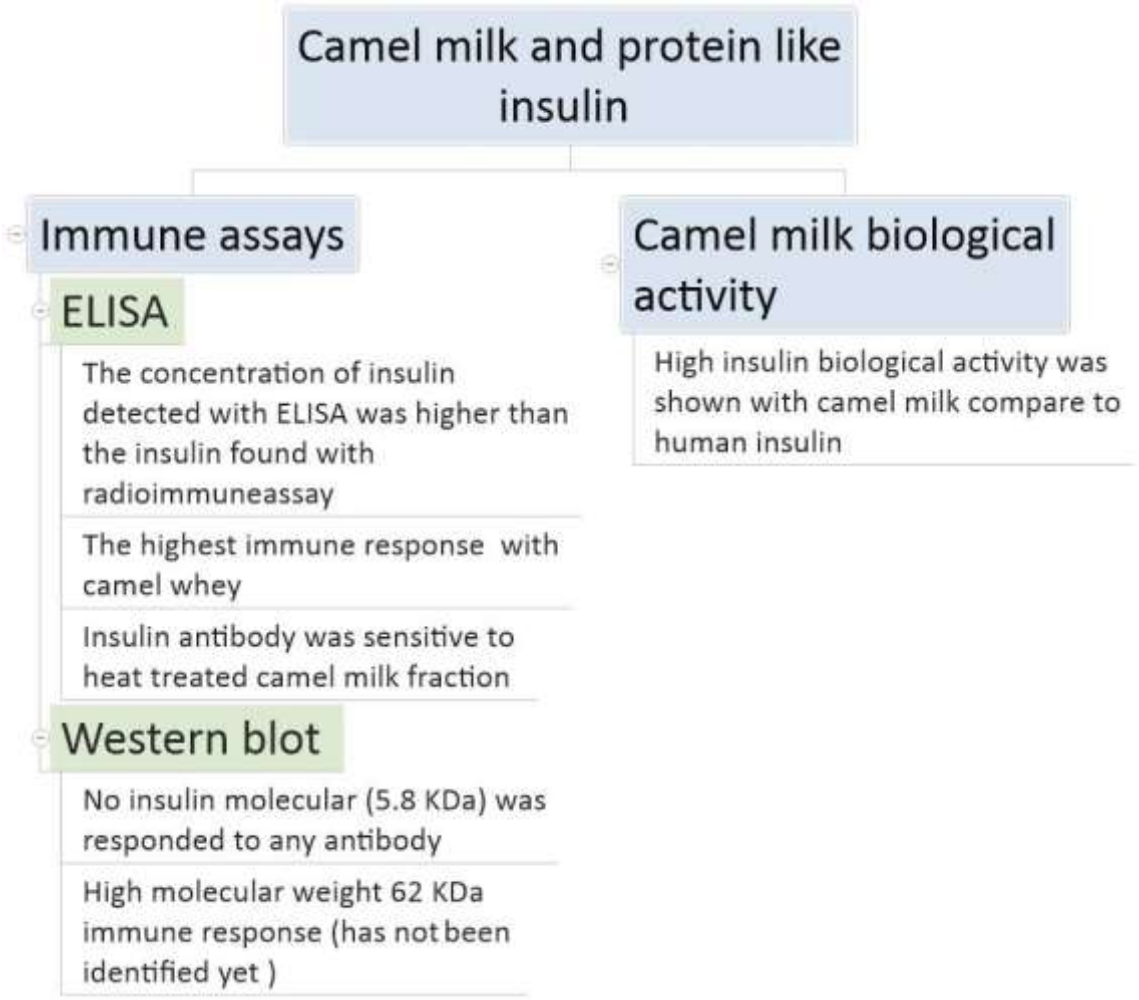

Figure 79 Conclusion for this chapter

In this study, the ELISA assay shows that insulin antibodies interact with antigens in camel milk. The reaction is more sensitive than that in the radio immune assay carried out by Agrawal (2005), and a 10-fold higher concentration of insulin was detected in camel milk. The ELISA results further show that the highest interaction of antigens is present in whey. It is also observable that the pasteurisation of camel milk reduced reactivity of the antigen. However, the western blot results indicate that the immune reaction obtained with ELISA was not due to reaction with the well-known size of 5.8 KDA, but with was 62KDa proteins. Immune precipitation leads to concentration of camel whey proteins, resulting in the detection of ~ 5.8 KDa band by SDS PAGE. This band, however, shows no immune reactivity with insulin antibodies. A PhD study conducted by Ismail (2013), also reported the absence of a 5.8 KDA band in camel milk as investigated by Western blot using anti human insulin antibodies, however the reactive band of 62 KDa has not reported.

However, a high molecular weight band (~ 62 kDa) that showed repeatable immune reactivity with anti-insulin antibodies reported in this study for the first time. As Bano et al. (1994) report, this size of molecule in human milk indicates that it could be a mammary-derived growth factor 1 (MDGF1). However, this hormone has not been fully identified (Playford et al. 2000). We postulate that the 62 kDa immune reactive band identified in camel milk could be a mammary derived growth factor that has some structural and biological similarities to insulin.

The in vitro assay for biological activity indicates camel milk insulin activity increases over time to become more active than the insulin control itself, which did not show any activity over a 48-hour incubation period. The failure to detect activity by the insulin control after 48 hours, leads to questioning the validity of the results, and requires further investigation.

The results of this study are not sufficient to confirm whether the camel milk contains insulin or has any biological activity. Moreover, no information is available regarding camel milk antigens that interact with anti-insulin in ELISA and western blot. It is thus recommended to extend the investigation into high molecule bands that react with anti-insulin antibodies. It is also important to explore the insulin and insulin-like biological

activity in camel milk**.** Continuing with the experimental study was not possible due to a lack of time and funding.

5.7 Novel results

This study achieved the following for the first time:

- A 10 fold higher concentration of insulin was measured with ELISA, compared to the work of Agrawal (2005), who detected insulin using a radioimmuneassay.
- A high concentration of n insulin immune reactive compounds was obtained in the whey fraction of camel milk.
- A decrease in the insulin concentration of camel milk following pasteurisation was noted.
- No insulin with molecular size of 5.8 KDa was detected by Western blot
- Repeatable immune reactivity was obtained for a 62 KDa molecular weight protein in camel milk.

5.8 Future work and recommendations

Recommendations for future work are as follows:

- Investigation of the effect of heat treatment on insulin activity in camel milk by using *in vitro* assays.
- Digestion of camel milk samples with enzymes (pepsin, trypsin and chymotrypsin) present in the human gut to investigate the effect of enzymes on insulin activity.
- Application of healthy liver cell lines to assess the insulin activity of camel milk on normal cells .

CHAPTER 6

CONCLUSIONS

A proximate analysis of camel skimmed milk powder indicated that protein concentrations were greater in camel milk than in bovine milk (37.36 ± 0.2% and 25± 0.7% respectively), and fat content was likewise greater (9.31±0.42% and 2±0.21% respectively). A comparison of chemical composition with previous study analysis indicated that protein concentrations were represented at a higher concentration in camel milk 33.93±.55% (Elamin & Wilcox, 1992). In the study conducted by Elamin and Wilcox (1992), the result was not compared with bovine milk compositions under the same conditions, however; furthermore, their results measured the effect of freeze-drying on camel milk's nutritional properties, confirming that the freeze-drying process had little effect on the fatty acid profile present in camel milk. The properties of lyophilized camel's milk remained basically unchanged when compared with fresh camel milk (Ibrahim & Khalifa, 2015). Proximate analysis of whey show protein concentration was higher in camel milk than bovine milk at 55 ±0.07 and 12.07 ± 0.7 respectively with fat concentration identified at 3±0.43 and 1.5 ±61 respectively. Camel casein was smaller in particle size in comparison to bovine casein. These differences in composition provide different properties to camel milk as compared to bovine milk fractions. Significantly higher fat concentrations in camel milk showed higher turbidity.

The spray dry processing of camel milk powder is similar to the processing of bovine milk powder, however the effects of drying on the physiochemical properties of both milk powders are different, though still comparable. Due to the differences in the heat stability of camel milk proteins as compared to bovine milk proteins, several researchers are now focusing on the functional properties of camel milk proteins and the issue of heat coagulation in camel milk. Camel milk powder may be used as a food ingredient due to the functional properties of its proteins which are different from those of bovine milk proteins, as indicated by viscosity and water holding capacity in this study.

Turbidity is the phenomenon in which a portion of a light beam passes through a liquid medium and is deflected from undissolved particles within that liquid (Daly, 2007). In the investigation of the two types of milk, camel and bovine, when both were heated to varying temperatures the turbidity remained stable between the two. This indicates a similarity of the size of particles and the amount of particles present between the two types of milk, regardless of temperature. The turbidity increase for whey

protein, however, was higher in bovine whey, indicating a disparity in the size and amount of particles between the two types of milk.

Solubility refers to the amount of a set substance that will dissolve into a given amount of a solvent (Smith, 2000). Liquid form solubility refers to the dissolution of that substance in liquid. When comparing the powdered camel milk and the powdered bovine milk in terms of solubility, the camel milk remained more soluble with heat treatment than the bovine milk. This indicates that, when heated, the mixture of powdered camel milk and water is more likely to retain its milk-like properties and the appropriate mixture of the two components. The solubility of the whey protein was comparative between bovine and camel milk, however, indicating a similarity in the proteins themselves.

Glycation occurs as a result of a typically covalent bonding of either a protein or a lipid molecule with a sugar molecule sans the controlling action of an enzyme (Uribarri et al., 2013). When comparing bovine and camel milk, glycation was higher in bovine milk. Glycation in bovine whey was higher than that of camel whey milk proteins, which is in line with previous findings and the increased glucose present therein.

SH is used to represent the thoil functional groups present in a diagram of chemical structure. Associations shown by thiols via hydrogen bonding, resulting in lower boiling points and a decreased solubility in water than other components of similar molecular weights like alcohols. The total SH groups in bovine milk were 10 umol/g while in camel milk it was 7 µmolar SH/g. This indicates that camel milk has a lower boiling point and a lower solubility than bovine milk. The ratio of free to total SH was likewise higher for camel milk at 0.3 µmolar, compared to bovine milk at 0.14 µmolar, leading to the absence of β Lactoglubolin in camel whey and serving as a means of indicating the reason for the decreased boiling point and lower solubility. There was a higher ratio of free to total SH for non-heated camel whey as compared to bovine whey.

When looking at the functional properties of camel milk and bovine milk, camel skim milk powder pre-heated to 90^{o}C produced the highest viscosity with the lowest water separation, a property that is attributed to the camel milk proteins present. The camel milk emulsion droplet sizes were stable with all heat treatments and were smaller

in size than bovine whey and bovine milk. The results indicated that the camel skim milk was more heat stable than bovine milk in terms of its functional properties. Ramet et al. (2001) postulated that the reason for this heat stability could be due to the absence of β-lactoglobulin and low κ- casein levels. The higher ratio of free to total SH groups (0.3 vs. 0.14 μmolar) indicated that more free SH groups are available to form aggregates during heating however, there was no significant increase in aggregate size (as turbidity and solubility were both stable), indicating the formation of small aggregates. This could be attributed to the lack of β-lactoglobulin preventing the formation of larger aggregates within itself or with κ- casein, as in heated bovine milk. The smaller camel milk aggregates could result in better emulsifying properties leading to a greater emulsion viscosity than present in bovine skim milk. The higher concentration of denatured whey protein in camel milk could also lead to higher water binding in emulsions at neutral pH levels when compared to bovine skim milk, resulting in higher viscosity. Looking to the functional properties of whey, within the two types of milk, camel whey was heat stable and provided stable emulsions at all temperatures and the camel whey emulsion droplet sizes were stable with all heat treatment and were smaller than that of bovine whey, further stressing the differences between the two, but offering up a reason as to the comparative nature of the two milks, due to the differences in viscosity.

The low turbidity and stable solubility of camel whey results in stable emulsions with smaller droplet sizes and less water separation, confirming the findings of Laleye et al. (2008) regarding the heat stability of camel whey in neutral oil/water emulsions. The higher free SH for camel whey and the sharp decline with heat treatment revealed heat sensitivity in camel whey, which is not expressed in the formation of large insoluble aggregates, the reason for its heat stability. In spite of this, the heat sensitivity does have an effect on the nutritional value of the milk, as indicated by the reduction in insulin like activity present in pasteurized camel milk, as described in Chapter 5. It was reported for the first time that the total SH in non-heated camel skim milk protein is lower than that of bovine skim milk, most likely as a result of a lack of β lactoglobulin in camel milk.

Camel milk was more heat sensitive than bovine skim milk, as indicated by the higher increases in turbidity, decreases in solubility, and a decrease in the total SH with an increase in free SH as compared to bovine skim milk. The camel milk was more heat

stable regarding its functionality due to the formation of smaller aggregates than are present in bovine milk. Liquid camel whey was more heat sensitive than bovine whey to heat treatment as regards to sulfhydryl groups, but was more stable concerning its solubility and functionality.

This investigation reports for the first time the superior emulsion viscosity enhancing water holding ability and oil droplet size reducing ability of heat-treated camel skim milk in comparison to heat-treated bovine skim milk in an oil in water emulsion with a pH 7. This investigation also reports the excellent heat stability at all temperatures of camel whey concerning emulsion (pH7) water holding, oil droplet size, and viscosity as compared to heat-treated bovine whey.

Milk gel was prepared to reach the best curt with camel milk. According to the physcomechal properties demonstrated in Chapter 2, camel milk was adjusted with agarose because of its acceptability for consumer consumption and its use as a well-known food in Saudi Arabia. Camel milk gel was applied with different heat treatment durations and fermented with several concentrations of GDL with the best time for adding GDL identified. These gels were developed to serve as a basis for the creation of fermented yogurt. This process consisted of the identification for the optimal heat treatment of milk mixture before acidification, which was established as 45 minutes at 90^{o}C using a 4% pre-heat-treated camel milk powder with 1% agarose and 4% GDL. The resultant experiment determined that camel milk forms lower viscosity gels with smaller coagulates than bovine milk as revealed by confocal microscopy. This lower viscosity gel serves to indicate an area of exploration for the creation of yogurt that will be more liquid in nature with a potential for greater consumer preference. The addition of pre-heat-treated skim milk powder significantly enhanced the viscosity and reduced syneresis in the acid gel, compared to the non-heat-treated control, indicating a need for heat in the yogurt making process for the appropriate consistency; to the knowledge of the researcher, this is the first time that such a fact has been reported. The addition of a pre-heat-treated whey powder did not significantly enhance the viscosity or reduce the syneresis of the yogurt, indicating the ability to increase the amount of protein present within the yogurt without a change to the viscosity, improving the overall health properties of the created yogurt without causing any adverse change to the compound. The inclusion of camel milk casein powder resulted in an increased viscosity of the yogurt, reduced the syneresis, and smoothed the texture of the gel, another reported first

in this area of organic chemistry. This serves to indicate a way to increase the overall smoothness of the resultant compound while having relatively little effect on the overall composition of the yogurt in terms of its comparative properties, bearing further investigation.

The completion of an SDS PAGE followed by Coomassie staining revealed the formation of proteins with a high molecular weight by disulphide bonding after 35 and 60 minutes of heating the acid gels and corresponded to an increase in the viscosity of the acid gel while the completion of an SDS PAGE followed by Ponceau staining revealed the formation of glycated proteins with a high molecular weight after 45 and 60 minutes of heating acid gels and corresponding to an increase of the viscosity of the acid gel. This indicates that the completion of the SDS PAGE and the application of heat, regardless of staining, results in the creation of proteins with higher molecular weights. Further investigation is warranted to determine whether this is the result of the SDS PAGE, the result of the application of heat, or the combination of both.

The completion of this study indicated that the best properties were achieved with heat-treated camel milk in terms of the creation of yogurt were as follows. Camel casein yogurt, prepared with Kefir and *L.bulgaricus + S.Thermophilus* provided the best texture, viscosity, pH balance, and syneresis for camel milk and casein samples when compared to the YOGO microbial culture. The results of the sensory evaluation indicated that the most desirable sample of those made was the camel milk and casein yogurt made with Kefir. The least desirable sample, however, was made with the YOGO microbial cultures. Yogurt prepared with casein reported the greatest desirability in comparison to the yogurt made with camel milk where both used the same microbial culture. There have been many studies published in regard to the creation of camel milk yogurt (Hashim, Khalil, & Habib, 2009; Abu-Tarboush, 1996; Edrees, 2013; Khalifa & Ibrahim, 2015). Of these, the report that is the most similar in terms of the ingredients used in the completion of this study was the study conducted by Khalifa and Ibrahim (2015). These ingredients are the easiest to obtain on a commercial level. In the completion of their study, the authors prepared camel milk yogurt through the addition of bovine gelatine E441, monoglycerides and diglycerides of fatty acid E471, guar gum E412, sodium carboxymethyl cellulose E446, modified starch E1422, and monoglycerides and triglycerides of fatty acid E471 to fresh camel milk to create the high viscosity and to prevent serum separation (Khalifa & Ibrahim, 2015). In this study,

however, seaweed agarose was the only thing applied as a yogurt stabilizer because the remainder of ingredients were camel milk fractions (skim milk or casein powder) reducing the number of E-ingredients necessary for the conduction of this research.

In this study, the ELISA assay shows that insulin antibodies interact with antigens in camel milk. The reaction is more sensitive than that in the radio immune assay carried out by Agrawal (2005), and a 10-fold higher concentration of insulin was detected in camel milk. The ELISA results further show that the highest interaction of antigens is present in whey. It is also observable that the pasteurisation of camel milk reduced reactivity of the antigen. However, the western blot results indicate that the immune reaction obtained with ELISA was not due to reaction with the well-known size of 5.8 KDA, but with was 62KDa proteins. Immune precipitation leads to concentration of camel whey proteins, resulting in the detection of ~ 5.8 KDa band by SDS PAGE. This band, however, shows no immune reactivity with insulin antibodies. A PhD study conducted by Ismail (2013), also reported the absence of a 5.8 KDA band in camel milk as investigated by Western blot using anti human insulin antibodies, however the reactive band of 62 KDa has not reported. A high molecular weight band (~ 62 kDa), however, showed repeatable immune reactivity with anti-insulin antibodies reported in this study for the first time. As Bano et al. (1994) report, this size of molecule in human milk indicates that it could be a mammary-derived growth factor 1 (MDGF1). However, this hormone has not been fully identified (Playford et al. 2000). We postulate that the 62 kDa immune reactive band identified in camel milk could be a mammary derived growth factor that has some structural and biological similarities to insulin.

The in vitro assay for biological activity indicates camel milk insulin activity increases over time to become more active than the insulin control itself, which did not show any activity over a 48-hour incubation period. The failure to detect activity by the insulin control after 48 hours, leads to questioning the validity of the results, and requires further investigation. The results of this study are not sufficient to confirm whether the camel milk contains insulin or has any biological activity. Moreover, no information is available regarding camel milk antigens that interact with anti-insulin in ELISA and western blot. It is thus recommended to extend the investigation into high molecule bands that react with anti-insulin antibodies. It is also important to explore the insulin and insulin-like biological activity in camel milk**.** Continuing with the experimental study was not possible due to a lack of time and funding. In spite of these

findings, however, other studies have been conducted to indicate that camel milk not only has sufficient levels of insulin present therein, the levels of insulin present in camel milk are great enough to aid in the prevention, and aid in the treatment of both type 1 and type 2 diabetes (Rasolt, 2014; Agrawal, Jain, Chopra, & Agarwal, 2011; Agrawal et al., 2003; Kochar et al., 2004; Mohamad et al., 2009; Al-Numair, 2011; Hassan, 2012; Romeih, Hamad, & Abdel-Rahi, 2011; Sboui et al., 2009; Sboui, Khorchani, & Djegham, 2012). This serves to indicate that the experiment conducted in this study may need to be repeated, as the presence of insulin in the camel milk should have been detected, according to previous studies.

During the course of this study, scientific data was presented as a basis for the development of three functional ingredients from camel milk:

1) Powder made from skimmed milk treated at 90°C. The knowledge of the degree of denaturation as regards to free and total sulfhydryl groups enable the scale up of manufacture for the product.

2) Powder made from camel milk whey. As it is heat stable as regards emulsification at neutral pH; the standard pasteurisation process for the liquid form would be sufficient. However, low pasteurisation intensity is required to maintain nutritional quality as regards to immune reactivity of insulin- like protein. The knowledge of the degree of denaturation as regards free and total sulfhydryl groups should enable the scale up of manufacture for both kinds of products.

3) Powder made from casein precipitated from camel skimmed milk. The product derived from milk that had been subjected to standard pasteurisation temperatures demonstrates good functional properties. The effect of the heat treatment of milk on precipitated casein still requires further investigation.

The feasibility of the production of these ingredients may be judged according to the camel milk supply information, which indicated that the “available world production of camel milk is officially put at 1.3 million tonnes, a tiny as compared to cow’s milk. However, a recent FAO/CIRAD/KARKARA workshop estimated global camel milk output as 5.3 million tonnes, although even this may be a conservative estimate. Lactating camels each produce between 1,000 and 12,000 litres of milk for anywhere between 8 and 18 months. The world’s biggest camel milk producer is Somalia, with

850,000 tonnes a year, followed by Saudi Arabia with 89,000 tonnes" (FAO, 2012, p. 1). While the camel milk market is an informal trade, it is not considered unreasonable to estimate this market at $10 billion dollars on a worldwide scale (FAO, 2012). In order to tap into such a market, however, it becomes necessary to overcome certain issues in the production, collection, processing, and marketing of this commodity.

The study offered up a scientific basis for the development of three products using the development of new treatments and new functional ingredients. These three products were:

1) **Oil in water emulsions at neutral pH**

   We report here for the first time on the excellent viscosity enhancing, water holding ability and oil droplet size reducing ability, of heat-treated (90°C) camel skimmed milk, in comparison with heat treated bovine skimmed milk in an oil in water emulsion with a pH of 7. This could be attributed to the smaller camel milk aggregates that result in better emulsifying properties leading to higher emulsion viscosity than bovine skimmed milk. The higher concentration of denatured whey protein in camel milk could lead to higher water binding in emulsions at neutral pH compare to bovine skimmed milk, resulting in higher viscosity. This investigation reports here for the first time on the excellent heat stability (at all temperatures) of camel whey, as regards to emulsion (pH 7), water holding, oil droplet size and viscosity, comparative to heat treated bovine whey. Preheated (90°C) camel skimmed milk powder resulted in yoghurt with significantly higher viscosity and reduced syneresis compared to bovine heat-treated skimmed milk powder, confirming the beneficial effect of heat treatment of camel skimmed milk in neutral emulsions. Camel casein powder also resulted in yoghurt with excellent viscosity, probably due to the small casein aggregates, high protein content and low lactose content. This would serve to have potential application in the creation of pasteurized culinary sauces and desserts.

2) **Yoghurt**

   A process was developed for preparation for a spoon able fermented yoghurt, enriched with camel milk proteins and containing only one stabiliser (seaweed agarose), reported here for the first time. Yoghurt enriched with Camel casein powder (8%) or preheated skimmed milk powder (8%) yogurt, or skimmed milk prepared with Kefir and *L.bulgaricus* + *S.Thermophilus* provided the best texture and sensory acceptability.

It is possible that the recipe can be further optimised by using blends of these two ingredients. The knowledge provided for producing a high viscous curd could be extended to cheese making.

3) **Nutritional whey**

Evidence of insulin like activity and heat sensitivity is reported in camel whey. This could lead to the development of whey products enriched with insulin like activity as high insulin immune reactivity activity has been found in heat sensitive camel whey, yet the characteristic 5.7 KDa insulin molecular weight band was not detected. However, a high molecular weight band (~ 62 kDa) that showed repeatable immune reactivity with anti-insulin antibodies is reported in this study for the first time. We postulate that the 62 kDa immune reactive could be a mammary derived growth factor that has some structural and biological similarities to insulin.

As there is an increasing market demand for diversified camel milk products, to the tune of an estimated 200 million potential customers on a worldwide scale, the creation of such products serves to have great potential in terms of commercial development. The results of this study have demonstrated the effects of heat-treatment on the physicochemical and functional properties of camel milk fractions. A novel spoon able yogurt enriched with camel milk proteins was developed and the evidence of insulin like proteins in camel whey was successfully demonstrated. It is believed that these results will contribute significantly to the further development and commercialization of camel milk and camel milk derived products. It is further believed that the underlying scientific knowledge presented herein has contributed significantly to the understanding of camel milk proteins.

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